ENGINEERING A PRO-APOPTOTIC BCG STRAIN TO IMPROVE EFFICACY OF THE CURRENT TUBERCULOSIS VACCINE

by

Ting Ting Alice Lau

B.Sc., The University of British Columbia, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

February 2017

© Ting Ting Alice Lau, 2017
ABSTRACT

Bacillus Calmette-Guérin (BCG), introduced almost 100 years ago, is the only vaccine designed to prevent tuberculosis (TB). BCG effectively protects newborns from meningeal TB yet fails to prevent adult pulmonary TB. In fact, TB kills 1.3 million people annually in areas where BCG vaccination is widely practiced. Thus, more efficient TB vaccines are urgently needed.

We and others have shown that BCG possesses the same virulence traits of Mycobacterium tuberculosis, in particular attenuation of essential macrophage functions such as phagosome maturation and antigen presentation. One of these studies revealed that defect in antigen presentation is largely due to down-regulation of the macrophage’s cysteine protease cathepsin S (CatS), which leads to prevention of MHC II molecule maturation and proper antigen presentation. Recent studies also suggested a potential role for cysteine proteases in the regulation of apoptosis, a key cellular process used by the macrophage to i) contain and process ingested bacteria and ii) facilitate cross-talk antigen presentation between the macrophage and dendritic cells.

To reverse the phenotype of vaccine-mediated macrophage attenuation, we engineered a novel BCG strain that expresses and secretes active CatS (rBCG-CatS). Since caspase-3 plays a central role in the execution of apoptosis, we also constructed a BCG strain that secretes an active form of caspase-3 (rBCG-C3).

Macrophages infected with either recombinant strain elicited a pro-apoptotic phenotype as indicated by increased levels of annexin V surface staining, PARP degradation, and caspase-3 cleavage compared to parental BCG. Furthermore, macrophage transcriptomic
profiling revealed that rBCG-CatS up-regulates key pro-apoptotic genes and down-regulates anti-apoptotic genes, which were further confirmed by RT-qPCR analyses. Consistent with these findings, mice vaccinated with rBCG-CatS or rBCG-C3 showed increased antigen-specific CD4+ and CD8+ T-cell responses, as well as enhanced cytokine production and proliferation upon ex vivo re-stimulation. Of particular note, immunogenicity responses from mice vaccinated with rBCG-C3 exceeded the effects observed with rBCG-CatS, demonstrating that induction of apoptosis is key to achieving high immunogenicity of TB vaccines.

Collectively, we have shown that by modifying BCG we can promote key host traits that confer high potential in improving efficacy of the TB vaccine.
PREFACE

Design of all research, data analysis, and manuscript preparation were completed with the assistance of Dr. Zakaria Hmama.

All experiments were designed and performed by the author with the following exception in Section 4:
Figures 18 and 19 were generated by Dr. Vijender Singh.

Parts of this thesis have been accepted for publication in a peer-reviewed journal:


In this study, I designed and performed all experiments except Figure 1 and Table 1, which were performed by Soualhine, H. The mouse immunogenicity experiments were designed with the assistance of Singh, V. I wrote the manuscript with assistance from Hmama, Z. This published work is located in Sections 3 and 6.1.

Important molecular biology tools used during my PhD studies were generated during a pre-doctoral training I performed in Dr. Hmama’s laboratory and were published in 2009:


In this study, I contributed to the design of and performed the experiments related to cloning of most of the entry and destination vectors. Sun, J. contributed to the development of work
shown in Figures 1, 2 and 5. Wang, X. and Liao, T.Y. A. contributed to the development of the work shown in Figures 3 and 4. The manuscript was written by Sun, J. and Hmama, Z. I edited the material and method section as well as the result and discussion sections.

Reprinted with permission from Elsevier.

*Co-first authors with equal contributions.

I will briefly refer to it in the Materials and Methods Section 2.4.2.

All animal experiments were performed in accordance with the UBC Animal Care Committee guidelines under the following protocols: A11-0247 and A16-0041.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ ii

PREFACE ........................................................................................................................................ iv

TABLE OF CONTENTS .................................................................................................................... vi

LIST OF TABLES ............................................................................................................................... x

LIST OF FIGURES ............................................................................................................................ xi

LIST OF ABBREVIATIONS ................................................................................................................ xiii

ACKNOWLEDGEMENTS .................................................................................................................... xix

CHAPTER 1: INTRODUCTION ...................................................................................................... 1

1.1 Tuberculosis .................................................................................................................................. 1
  1.1.1 Scope and global impact of the disease .................................................................................. 1
  1.1.2 Mtb Life cycle, TB diagnosis, and treatment ....................................................................... 2
  1.1.3 Current vaccine, its shortcomings, and proposed solutions ............................................... 8

1.2 Apoptosis ..................................................................................................................................... 11
  1.2.1 Overview ............................................................................................................................... 11
  1.2.2 Key players and pathways ...................................................................................................... 11
  1.2.3 Dysregulated apoptosis and pathology .................................................................................. 14
  1.2.4 Role of apoptosis in TB pathogenesis ................................................................................... 15
  1.2.5 Role of apoptosis in TB immunity .......................................................................................... 17

1.3 Caspase-3 ................................................................................................................................... 18
  1.3.1 Caspase-3 processing and activation ..................................................................................... 18
  1.3.2 Execution of apoptosis by caspase-3 ..................................................................................... 19
1.4 Cathepsin S.................................................................................................................. 20
  1.4.1 Overview of cysteine cathepsins ........................................................................... 20
  1.4.2 Overview of MHC presentation pathway ......................................................... 21
  1.4.3 Role of CatS in MHC II maturation ................................................................. 25
  1.4.4 Lysosomal pathway of apoptosis ..................................................................... 27
  1.5 Aim of study .......................................................................................................... 29

CHAPTER 2: MATERIALS AND METHODS ................................................................... 31
  2.1 Reagents and chemicals ....................................................................................... 31
  2.2 Antibodies ............................................................................................................. 31
  2.3 Cell culture ............................................................................................................ 32
    2.3.1 Cell line maintenance and propagation ....................................................... 32
    2.3.2 BMDM culturing ............................................................................................. 32
    2.3.3 Infection ........................................................................................................... 33
  2.4 Bacteria .................................................................................................................. 33
    2.4.1 Mycobacterial strains and growth conditions .............................................. 33
    2.4.2 Construction of mycobacterial Destination Vectors .................................... 33
    2.4.3 rBCG-CatS and rBCG-C3 constructions ...................................................... 35
    2.4.4 Mycobacteria transformation ........................................................................ 36
    2.4.5 Mycobacteria expression ............................................................................. 36
  2.5 Microarray and RT-qPCR study ............................................................................ 36
  2.6 Apoptosis analysis ............................................................................................... 38
    2.6.1 Annexin V microscopy .................................................................................... 38
    2.6.2 Flow cytometry ............................................................................................... 39
2.6.3 Western analysis .................................................................................................................. 39
2.7 Murine immunogenicity studies .............................................................................................. 40
  2.7.1 Mouse immunization and splenocyte harvesting ................................................................. 40
  2.7.2 Tetramer staining ................................................................................................................. 40
  2.7.3 CFSE cell proliferation assay .............................................................................................. 41
  2.7.4 Intracellular cytokine staining ............................................................................................ 41
2.8 Statistical analyses ................................................................................................................... 42

CHAPTER 3: RECOMBINANT BCG EXPRESSING CATHEPSIN S IMPROVES HOST
CELL APOPTOSIS AND VACCINE IMMUNOGENICITY ......................................................... 43
  3.1 Background ........................................................................................................................... 43
  3.2 Global macrophage transcriptome profiles in response to BCG expressing Cathepsin S
.................................................................................................................................................. 44
  3.3 Chromosomal expression of human active CatS in BCG ..................................................... 46
  3.4 rBCG-CatS induces the expression of macrophage pro-apoptotic genes ......................... 48
  3.5 Recombinant Cathepsin S protein induces active-caspase 3 ............................................. 51
  3.6 BCG prevents macrophage apoptosis while rBCG-CatS induces it ................................. 52
  3.7 CatS expression in BCG improves its immunogenicity ....................................................... 56

CHAPTER 4: RECOMBINANT BCG EXPRESSING CASPASE-3 FURTHER IMPROVES
VACCINE IMMUNOGENICITY .................................................................................................. 64
  4.1 Background ........................................................................................................................... 64
  4.2 Characterization of rBCG-C3 ............................................................................................. 64
  4.3 Human and murine cell lines infected with rBCG-C3 showed increased level of
apoptosis ........................................................................................................................................ 66
4.4 Improved immunogenicity of rBCG-C3 infected mice................................. 68

CHAPTER 5: DISCUSSION................................................................................. 76

5.1 Expression of CatS in BCG converts it into a more immunogenic vaccine .......... 76
5.2 Proposed mechanism of cathepsin S promoting apoptosis .............................. 80
5.3 Benefits of a recombinant BCG strain expressing executioner caspase .......... 81

CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS .............................. 85

6.1 Conclusion.............................................................................................. 85
6.2 Future directions..................................................................................... 85

REFERENCES ............................................................................................... 87
LIST OF TABLES

Table 1. List of primers used in RT-qPCR study................................................................. 38

Table 2. Macrophage transcriptome in response to rBCG-hcs ........................................ 46
**LIST OF FIGURES**

Figure 1. The life cycle of Mtb. ................................................................. 4

Figure 2. Overview of apoptosis pathways ................................................. 12

Figure 3. Model of the MHC alpha/beta hetero-dimer associated with Ii chain .......... 23

Figure 4. Invariant chain degradation .......................................................... 24

Figure 5. The MHC II presentation pathway ............................................... 25

Figure 6. Microarray analysis of macrophage transcriptome in response to BCG and rBCG-hcs ................................................................................. 45

Figure 7. Construction and characterization of recombinant BCG expressing CatS ....... 48

Figure 8. Expression level of select apoptosis genes in RAW 264.7 cells infected with BCG or rBCG-CatS ........................................................................................................... 50

Figure 9. CatS-coated beads induce active caspase-3 expression in RAW 264.7 .......... 52

Figure 10. BCG does not induce activate caspase-3 in the cell ......................... 53

Figure 17. BCG inhibits caspase-3 activation in the presence of staurosporine in RAW 264.7 cells .................................................................................................................. 54

Figure 12. rBCG-CatS induces cellular apoptosis ............................................ 56

Figure 13. Ag85+ CD4+ T-cells generated in C57BL/6 mice immunized with rBCG-CatS. 58

Figure 14. CD4+ T-cells proliferation upon *ex vivo* re-stimulation from C57BL/6 mice immunized with rBCG-CatS. .............................................................................. 59
Figure 15. Intracellular cytokine expression of IFN\(\gamma\) upon \textit{ex vivo} re-stimulation from C57BL/6 mice immunized with rBCG-CatS................................................................. 60

Figure 16. Intracellular cytokine expression of TNF\(\alpha\) upon \textit{ex vivo} re-stimulation from C57BL/6 mice immunized with rBCG-CatS................................................................. 61

Figure 17. Intracellular cytokine expression of IL-2 upon \textit{ex vivo} re-stimulation from C57BL/6 mice immunized with rBCG-CatS................................................................. 63

Figure 18. Construction and characterization of recombinant BCG expressing active caspase-3 ................................................................................................................................. 65

Figure 19. rBCG-C3 induces cellular apoptosis \textit{in vitro}................................................................. 67

Figure 20. Immunogenicity studies in C57BL/6 mice................................................................. 69

Figure 21. Immunogenicity rBCG-CatS vs rBCG-C3................................................................. 73

Figure 22. Schematic of BCG surface decoration approach................................................................. 84
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AATF</td>
<td>apoptosis antagonizing transcriptional factor</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa Fluor</td>
</tr>
<tr>
<td>Ag85A</td>
<td>antigen 85 A</td>
</tr>
<tr>
<td>Ag85B</td>
<td>antigen 85 B</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>Akt1</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
</tr>
<tr>
<td>ALPS</td>
<td>autoimmune lymphoproliferative syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptosis protease activating factor</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl2-associated agonist of cell death</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting-domain death agonist</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2-like protein 11</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow derived macrophage</td>
</tr>
<tr>
<td>BMRC</td>
<td>British Medical Research Council</td>
</tr>
<tr>
<td>BMT</td>
<td>bone marrow transplantation</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>C3</td>
<td>caspase-3</td>
</tr>
<tr>
<td>CAD</td>
<td>caspase-activated DNAse</td>
</tr>
<tr>
<td>Cat</td>
<td>cathepsin</td>
</tr>
<tr>
<td>CatS</td>
<td>cathepsin S</td>
</tr>
<tr>
<td>CatX</td>
<td>cathepsin X</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CD62L</td>
<td>L-selectin</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyld ester</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestine alkaline phosphatase</td>
</tr>
<tr>
<td>CLIP</td>
<td>class II-associated Ii peptide</td>
</tr>
<tr>
<td>ConA</td>
<td>concavalin A</td>
</tr>
<tr>
<td>cTEC</td>
<td>cortical thymic epithelial cell</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DAP</td>
<td>death-associated protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signaling complex</td>
</tr>
<tr>
<td>DLI</td>
<td>donor lymphocyte infusion</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DST</td>
<td>drug susceptibility test</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>eIF2alpha</td>
<td>eukaryotic initiation factor-2alpha</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESAT6</td>
<td>6 kDa early secretory antigenic target</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FasR</td>
<td>Fas receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GOI</td>
<td>gene of interest</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>hcs</td>
<td>human cathepsin S</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA-DM</td>
<td>human leukocyte antigen DM</td>
</tr>
<tr>
<td>hly</td>
<td>listeriolysin</td>
</tr>
<tr>
<td>HlyA</td>
<td>hemolysin A</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>ICAD</td>
<td>inhibitor of CAD</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IGRA</td>
<td>interferon gamma release assay</td>
</tr>
<tr>
<td>Ii chain</td>
<td>invariant chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani media</td>
</tr>
<tr>
<td>LCM</td>
<td>L-cell conditioned media</td>
</tr>
<tr>
<td>LLO</td>
<td>Listeriolysin O</td>
</tr>
<tr>
<td>LLOMe</td>
<td>L-leucyl-L-leucine methyl ester</td>
</tr>
<tr>
<td>LMP</td>
<td>low molecular weight polypeptide</td>
</tr>
<tr>
<td>M6PR</td>
<td>mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>myeloid cell leukemia 1</td>
</tr>
<tr>
<td>MDR</td>
<td>multi-drug resistant</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>mitochondrial membrane permeabilization</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MPT</td>
<td>mitochondrial permeability transition</td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>NAAT</td>
<td>nucleic acid amplification test</td>
</tr>
<tr>
<td>NDI</td>
<td>N-dodecylimidazole</td>
</tr>
<tr>
<td>Nef</td>
<td>negative regulatory factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute of Allergy and Infectious Diseases</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NTM</td>
<td>non-tuberculouse mycobacteria</td>
</tr>
<tr>
<td>nuoG</td>
<td>NADH ubiquinone oxidoreductase chain G</td>
</tr>
<tr>
<td>OADC</td>
<td>oleic acid albumin dextrose catalase complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PERK</td>
<td>double-stranded RNA-activated protein kinase-like ER kinase</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PKCδ</td>
<td>protein kinase C delta</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethane sulfonyl fluoride</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylinerine</td>
</tr>
<tr>
<td>Puma</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>rBCG</td>
<td>recombinant BCG</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline tween</td>
</tr>
<tr>
<td>T&lt;sub&gt;cm&lt;/sub&gt;</td>
<td>central memory T-cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>TEC</td>
<td>thymic epithelial cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;em&lt;/sub&gt;</td>
<td>effector memory T-cell</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper 1 cell</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR1</td>
<td>tumor necrosis factor receptor 1</td>
</tr>
<tr>
<td>TNFR2</td>
<td>tumor necrosis factor receptor 2</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TST</td>
<td>tuberculin skin test</td>
</tr>
<tr>
<td>ureC</td>
<td>urease C</td>
</tr>
<tr>
<td>WBL</td>
<td>whole (BCG) bacterial lysate</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XDR</td>
<td>extensively-drug resistant</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-chromosome-linked inhibitor of apoptosis</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

The work of this thesis could not have been accomplished without the unyielding support, guidance, and expertise from my supervisor Dr. Zakaria Hmama. He introduced me to the world of scientific research, and was there during every step of the journey to watch me grow.

I am grateful for my committee members, Dr. Yossef Av-Gay and Dr. Vincent Duronio, who have provided me with insightful ideas and comments for my work throughout the years.

I would like to thank all the past and present members of the Hmama lab, with special thanks to Dr. Vijender Singh for his intellectual inputs. I also appreciate all the friends I have acquired throughout my research journey, and of course all the friends who have remained by my side on this long and winding road.

I would like to acknowledge the financial support from TB Vets, Canadian Institute of Health Research (CIHR), BC Lungs Association, and the University of British Columbia.

Lastly, but perhaps most importantly, I must thank my parents and my husband for their unconditional love and support. Without them by my side, this doctoral degree would not have been possible.
CHAPTER 1: INTRODUCTION

1.1 Tuberculosis

1.1.1 Scope and global impact of the disease

Tuberculosis (TB), originally known as “consumption”, an antique term used to describe wasting away of the body, is a disease characterized by a chronic cough with blood-containing sputum, chest pain, fever, night sweats, and weight loss [1]. It was not until 1882, when German physician Robert Koch discovered the causative tubercle bacillus, *Mycobacterium tuberculosis* (Mt), that the name “tuberculosis” began to be used [2].

TB has been documented as far back as 3500 BC by ancient Egyptians, where osseous changes were found in the spines of mummies, indicated spinal TB infection [3]. Scourging through Europe and North America during the 18th and 19th centuries, the TB epidemic claimed up to 1,000 lives per 100,000 people per year [4]. Initially a romanticized condition among the arts [5], TB later developed a stigma associated with poverty and fear of transmission, that often caused delays in seeking treatment [6]. The discovery of streptomycin in 1944, along with massive BCG vaccination and the development of improved public health regimen, significantly reduced the global burden of TB disease. Unfortunately, this victory was quickly crushed with the rise of drug-resistant Mt strains in the 1980s, which made TB again out control, especially in developing countries. Such a dramatic situation urged the World Health Organization (WHO) to declare TB a global health emergency in 1993 and, as of today, TB remains the number one killer in infectious diseases by a single pathogen worldwide [1]. In 2014, 9.6 million people developed TB disease and 1.5 million perished from it [1]. Although >95% of TB mortality occurs in low to
middle income countries, a growing concern stems from TB being the leading killer among 
HIV-positive people worldwide [1]. In fact, having a compromised immune system drives up 
the risk of developing TB by 20-30-fold to the point that 1 in 3 deaths from AIDS is due to 
Mtb co-infection [1].

On the other hand, the HIV population contributes significantly to the spread of drug-
resistant TB to the general population [7]. Multi-drug resistant (MDR) TB is defined by 
resistance to at least isoniazid and rifampicin [8]. In addition, resistance to any 
fluoroquinolone and at least one of three second-line drugs (i.e. amikacin, kanamycin, or 
capreomycin) is deemed to be extensively-drug resistant (XDR) [9]. Global burden of MDR-
TB was estimated to be 5% of the total number of TB cases [1]. Out of these MDR-TB 
cases, approximately 9% develop XDR-TB, as reported by 100 countries [1].

1.1.2 Mtb life cycle, TB diagnosis, and treatment

TB is highly contagious; an infection can be established from as little as a single 
bacterium [10]. The bacilli are expelled as droplet nuclei into the environment by an 
individual with active disease, and these droplets are small enough to remain airborne for 
several hours to be inhaled by the next unsuspecting person [10]. Inhaled bacteria are 
phagocytized by alveolar macrophages, which triggers a local pro-inflammatory response 
and allows the infected cells to invade the subtending epithelium (Fig. 1). It takes 8-12 days 
post-infection before resident lung dendritic cells (DCs) become activated and migrate to the 
lung’s draining lymph nodes and activate naïve T-cells [11]. 14-17 days post-infection, 
antigen-specific Th1 cells are then able to arrive in the lungs [11]. During this time, Mtb is 
free to grow and modulate the environment to establish itself in a suitable niche [12].
Activated macrophages signal for the recruitment of fresh mononuclear cells from nearby blood vessels, providing new grounds for the spread of infection [10]. The infection quickly becomes extensively vascularized (within a few days) [13], and starts to form the hallmark TB structure, the granuloma [10]. Over the next few weeks, lymphocytes are recruited to the structure but are segregated by a fibrous cuff of extracellular matrix material, which encases the macrophages that have differentiated into epithelioid cells, multi-nucleated giant cells and foam cells filled with lipid droplets [10,13]. When the granuloma is presented in this “balanced state”, the disease is contained and considered inactive. Many factors in an individual’s life can trigger the onset of active TB. The progression of disease is characterized by a loss of vascularization [10], and an increase in foamy macrophages that leads to an accumulation of caseous debris in the granuloma center [14], which later becomes hypoxic [15] and necrotic [10]. The ultimate fate of the granuloma is rupture and collapse into the lungs and escape of viable bacilli into the airway, spreading the infection via productive coughs [10].
Figure 1. The life cycle of Mtb. The bacillus is inhaled into the airway and taken up by alveolar macrophages, triggering pro-inflammatory signals that recruit lymphocytes and promote vascularization. The granuloma is formed and maintained when the individual is not in an active form of disease. However, when the structure collapses, infectious bacilli are released into the airway and active TB ensues. Russel DG et al. Nat Immunol. 2009. 10(9):943-948. Reprinted with permission from Nature Publishing Group.
Diagnosis of patients with active TB disease typically involves assessment of clinical symptoms in combination with molecular and culture-based laboratory tests [16]. Clinical symptoms of TB disease are defined by a persistent and productive cough, fever, night sweats, and weight loss [16,17]. In addition to manifestation of disease symptoms, proper clinical diagnosis of active TB would include (1) chest radiography, (2) sputum smear microscopy, (3) mycobacterial culture and phenotypic drug susceptibility tests (DST), and (4) nucleic acid amplification tests (NAAT) [17].

Chest radiography is often the first tool used to examine an individual suspected of having active TB, but this method is limited by a sensitivity of only 70-80% and a poor specificity of 60-70% [17]. Complicating this further, there is very poor agreement among readers on the interpretation of the X-rays, leading to uncertainties in the diagnosis of active disease [17,18]. Despite this, chest radiography remains an important part of TB diagnosis, but requires follow-up microbiological tests for confirmation.

In this regard, sputum smear microscopy is the most widely used method to test for active TB, due to its high speed, low cost, and ability to identify highly infectious TB patients [17,19,20]. This method is dependent on the type of specimen, patient population, choice of stain, and experience of the microbiologist, thus resulting in a sensitivity of 20-80% that is highly variable [17,21-23]. Furthermore, in low TB incidence countries, a positive smear could be confounded by non-tuberculous mycobacteria (NTM) [17]. Nevertheless, in many low-resource, TB endemic countries, sputum smear microscopy is often the sole method of TB diagnosis used to supplement clinical symptoms [16].
Given the deficiencies of sputum smear microscopy, high-resource countries including Canada subjects the same specimen to culturing regardless of the sputum smear results [17]. Mycobacterial culture methods are the current gold standard for detection of active TB, and are considered to be the most sensitive [17,19,20]. Depending on the culture method (i.e. solid or liquid media) and number of bacteria in the inoculum, culture results typically take between 2-8 weeks [17]. While occasionally false positives are observed, it is often due to cross-contaminations in the laboratory. However, in such cases, longer than usual detection time and/or fewer colonies would raise suspicions and further tests would be conducted to confirm results [17]. Beyond the definitive diagnosis of active TB, culturing can also provide valuable information on species identification, drug-susceptibility, and enables use of the culture for further molecular work [17,20].

In addition to culture-based diagnosis of active TB, molecular tests are now available to produce a much faster result than conventional culture methods. Indeed, Nucleic Acid Amplification Tests (NAAT), which utilizes the polymerase chain reaction (PCR) technique are used to quickly diagnose TB and to detect drug resistance [17]. While the commercial NAATs have high sensitivity to detect TB (>95%), this is limited to sputum smear-positive samples, as sensitivity drops to 50%-70% in smear-negative/culture-positive samples and extrapulmonary specimens [17,24-26]. Therefore, a negative NAAT result should not be used to rule out the possibility of active TB [17]. Moreover, NAATs require sophisticated laboratory infrastructure and highly skilled technicians, as any contaminating DNA at the test site would compromise the results [17].

The mycobacterial culture (phenotypic DST) and the NAAT (genotypic) methods are further used in conjunction for the diagnosis of drug-resistant TB [17,19,27-29]. DST of
mycobacterial cultures provides phenotypic results within 4-14 days and 4-21 days for first- and second-line drugs, respectively [27,28]. Genotypic methods such as NAATs are used to detect mutations associated with drug resistance. While commercially available NAATs (line-probe assays and the Xpert MTB/RIF test) are quick and relatively specific and sensitive, they are only useful for detection of resistance to rifampicin and isoniazid [17]. Thus, culture DST is recommended to confirm drug-resistance TB diagnosis.

Immune-based methods for diagnosis of TB include serological tests, tuberculin skin test (TST), and interferon gamma (IFN\(\gamma\)) release assays (IGRAs) [17]. However, the use of serological tests is strongly discouraged by the WHO, as they are unreliable and expensive [17,30-32]. Similarly, the use of TST and IGRAs are also not recommended for diagnosis of active TB, as neither can differentiate between latent TB infection (LTBI) and active TB [17,33]. However, these immunodiagnostic tools can serve as indicators for pediatric tuberculosis, but only in conjunction with other standard TB diagnosis methods discussed above [17].

When the first TB patient was successfully treated by streptomycin monotherapy in 1944 [34], the world thought that Mtb had met its demise. In 1948, the British Medical Research Council (BMRC) conducted the first large-scale clinical trial for the use of streptomycin in TB patients and concluded that streptomycin monotherapy was efficacious and significantly reduced immediate morality [35,36]. However, in the same year, Crofton and Mitchison reported the first streptomycin resistance in Mtb [37]. Over the next few decades, new drugs have been introduced and different combinations of drugs for treatment have been extensively analyzed [36]. This led to the current antibiotic regimen for TB, which is a combination of four drugs (rifampin, isoniazid, pyrazinamide, ethambutol or
streptomycin) for 6 months. While a chemotherapy regimen is in place, the long treatment duration and unpleasant side effects result in compliance issues, which continues to contribute to the emergence of drug-resistance Mtb strains [38]. In addition, Mtb is also prone to spontaneous mutations that occur at predictable rates [39]. This is compounded by the fact that only a few new antibiotics have entered the market in recent decades, due to the declining interest in TB drug development by major pharmaceutical companies [40]. For all of the reasons above, many investigators believe that vaccination would be the most effective and cost-conscious preventive method to control TB globally [41]. Unfortunately, the current and only TB vaccine available, Bacillus Calmette-Guérin (BCG) has a limited efficacy and needs to be replaced or urgently improved.

1.1.3 Current vaccine, its shortcomings, and proposed solutions

One of the most widely administered vaccines worldwide [42], BCG was first isolated in 1921 by continuous in vitro passaging of the bovine TB bacillus, M. bovis, until it has lost its virulence in calves and guinea pigs [42]. While BCG has proven itself worthy in preventing disseminated diseases, especially miliary and meningeal TB in children [43], its efficacy in pulmonary TB is highly variable [44,45]. There are 3 main hypotheses to why BCG has variable efficacy. (1) BCG has become overly attenuated through repeated passaging in culture and modern preparations are inadequate in sustaining its potency [46], (2) unless vaccinated at birth, exposure of infants to environmental strains of mycobacteria could lead to tolerance [47-49], and (3) clearance of BCG in some populations may occur prior to development of a protective immune response [10]. In addition, and perhaps most importantly, the problem lies in BCG’s ability to mimic virulent mycobacterial strains in
blocking essential functions of antigen presenting cells (APC), such as phagolysosome fusion [50-52], antigen presentation [53,54] and its inability to induce apoptosis [55,56].

To further complicate the issue, the BCG we use to vaccinate against TB, is in fact inclusive of many substrains [57]. With genomic approaches, it was discovered that the substrains are different from each other, despite having been evolved from the same original BCG used in 1921 [57]. Currently, there are five main strains being used worldwide accounting for more than 90% of vaccinations – the Pasteur 1173 P2, the Danish 1331, the Glaxo 1077 (derived from the Danish strain and therefore grouped as one main strain), the Tokyo 172-1, the Russian BCG-I, and the Moreau RDJ strains [58]. While these differences certainly translate to variable adversity, their efficacy in vaccinated individuals remain hotly debated [58-60]. Horwitz et al. conducted a study comparing the efficacy of different BCG strains in the guinea pig model of pulmonary TB [59]. Specifically, they took timeline into account and compared evolutionarily early strains and evolutionarily late strains. The study concludes that late strains are not less potent than early strains, and denied that strain variability is a major contributing factor to BCG vaccine trial outcomes [59]. In contrast, Zhang et al. correlated that strain virulence is proportional to protective efficacy, at least in the murine model [60]. In addition to genetic differences between strains, the genetic differences within some strains can lead to major differences in the characteristics of the BCG vaccine produced from different manufacturer, and in some cases, even between batches from a single manufacturer [61]. Therefore, it is important for researchers to take into account the particular strain and characteristics of that strain they are working with, while attempting to improve the vaccine.
Since the realization that the BCG vaccine urgently requires an upgrade, there have been many proposals on improvement strategies, or perhaps how it may be replaced entirely. In general, the vaccines in working can be divided into three groups – (1) prophylactic, (2) post-exposure, and (3) therapeutic [62]. Within the prophylactic category, efforts have been focused on creating a recombinant BCG (rBCG), or an attenuated Mtb strain obtained through deletion of genes in metabolic pathways required for survival or full virulence [63-65], or using viral delivery systems encoding Mtb antigens or protein subunits [66-70]. In order to overcome the limitations of BCG, some investigators have exerted efforts towards novel rBCG strains that allow for increased antigen release into the cytosol from its phagosomal milieu [71,72]. Others opted for subunit vaccines based upon immune-dominant antigen delivery using viral vectors dedicated to boost the effect of BCG [73,74]. This strategy is known as the prime-boost strategy [73,74].

Improvement of BCG is attempted by introducing strongly immunogenic and specific Mtb antigens, or by over-expressing those already made by BCG, aiming to broaden the immune response [75,76]. Currently, only one recombinant BCG vaccine is prevailing in clinical trials (phase IIa) – the VPM1002 vaccine, developed at the Max Planck Institute for Infection Biology in Berlin, Germany. This live rBCG is an urease negative strain (ureC gene deletion) expressing listeriolysin O (LLO) (encoded by hly gene) from Listeria monocytogenes [71,72]. LLO is expected to perforate BCG phagosome, thereby allowing leakage of antigens into the cytoplasm and induction of apoptosis (by a mechanism yet to be identified) [77,78], whereas the absence of ureC allows pronounced acidification of the phagosome and thus creating the optimum biological pH for LLO activity [79-81]. This
paves the way to strategies that promote exposure of immunogenic antigens to lymphocytes along with apoptosis induction.

1.2 Apoptosis

1.2.1 Overview

Apoptosis, derived from the Greek meaning of “falling off,” as leaves often do from a tree [82], describes a fundamental cellular process known as “programmed cell death”. This process occurs in multicellular organisms in a highly organized and energy-dependent fashion [83]. Apoptotic cells are morphologically characterized by membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and maintenance of an intact plasma membrane until late stages of the process [84]. This is in contrast to necrosis, which is a disorganized process that ends in traumatic cell death due to acute cellular injury. Necrosis is morphologically identified with cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles, and moderate chromatin condensation [84]. Apoptosis is highly organized and is essential in an organism’s lifecycle. It is a vital process involved in embryonic development, normal cell turnover, and proper development and functioning of the immune system [83]. For example, during embryogenesis, fingers and toes are formed once the cells in-between undergo apoptosis, and roughly 50-70 billion cells die due to apoptosis in a human adult on a daily basis [85,86].

1.2.2 Key players and pathways

Apoptosis is widely known to be initiated through one of three pathways: the Intrinsic, Extrinsic, or the Perforin/Granzyme pathways (Fig. 2). While all pathways (except granzyme A) converge at the executioner caspase, caspase-3, each is initiated by different stimuli. The
intrinsic pathway, as the name suggests, occurs due to internal injury to the cell (e.g. cell stress). The extrinsic and perforin/granzyme pathways happen due to external stimuli such as kill signals from other cells (e.g. cytotoxic T-lymphocytes) or extracellular insults. When all 3 pathways converge at the execution step (caspase-3 activation), this results in DNA fragmentation, cross-linking of protein and protein degradation, formation of apoptotic bodies, and expression of ligands to signal for engulfment by phagocytic cells [83].

**Figure 2. Overview of apoptosis pathways.** This illustration summarizes the key players and sequence of events involved in each of the 3 apoptosis pathways, and the morphological changes that follows. Elmore S. Toxicol Pathol. 2007. 35:495-516. Reprinted with permission from SAGE Publications.

*Intrinsic pathway.* Previously exclusively considered to be the cell’s powerhouse, the mitochondrion was discovered to have a second function in the 1990s – the control of cell death [87]. The intrinsic pathway is initiated by intracellular signals that act either in a positive or negative fashion [83]. Negative stimuli involve the loss of apoptotic suppression;
for example, via the unavailability of growth factors, hormones and cytokines [83]. Positive stimuli include insults to the cell such as radiation, toxins, hypoxia, and viral infections [83]. Ultimately both stimuli result in the mitochondria losing structural integrity, leading to an opening of the mitochondrial permeability transition (MPT) pore, and therefore pro-apoptotic proteins that are normally sequestered in the intermembrane space get released into the cytosol [83,88]. Mitochondrial permeability is controlled by the symphony of pro- and anti-apoptotic Bcl-2 family proteins [89]. The pro-apoptotic members Bax and Bak form pores in the mitochondrial outer membrane [90]. This action is opposed by anti-apoptotic members including Bcl-2, Bcl-XL, and Mcl-1 [90]. Bax and Bak activities can also be mediated by the truncated, active version of Bid, called t-Bid [90]. T-Bid can be produced by a number of proteases, but also by caspase-8, therefore creating a cross-talk between extrinsic and intrinsic pathway [90]. One of the proteins released from the mitochondrion is cytochrome c, which then forms the “apoptosome” by recruiting apoptosis protease activating factor (Apaf-1) and procaspase-9 [91,92]. Procaspsase-9 gets activated by dimerization and leads to caspase-3 activation.

**Extrinsic pathway.** The extrinsic pathway requires transmembrane receptor-mediated interactions [83]. These death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily [93]. Members of this family have cysteine-rich extracellular domains and a cytoplasmic domain that contains the “death domain” [94]. The death domain transduces extracellular information to the inside of the cell to initiate apoptosis. The best studied receptor-ligand pairs are FasL/FasR and TNFα/TNFR1 [83]. Signal transduction begins with clustering of the receptors upon binding with the homo-trimers of the ligands on the cell surface [83]. When the death domains on the intracellular face of the receptors are
aggregated, cytoplasmic adapter proteins containing corresponding death domains are recruited [83]. This results in the recruitment of FADD, which then associates with procaspase-8 via dimerization of the death effector domain [83]. Here, a death-inducing signaling complex (DISC) is formed and procaspase-8 becomes activated via auto-catalytic activities [95]. Similar to the intrinsic pathway, activated caspase-8 proceeds to recruit and activate caspase-3.

**Perforin/Granzyme pathway.** The perforin/granzyme pathway induces apoptosis via granzyme A or granzyme B [83]. This pathway is used by cytotoxic T-lymphocytes (CTLs) to kill tumor or viral-infected cells [83]. CTL first secretes the transmembrane pore-forming molecule perforin into the target cell [96], then the serine proteases granzyme A and B are injected into the cell to exert their functions [83]. While granzyme B can cleave procaspase-10 at aspartate residues to aid in its activation, it can also directly activate caspase-3 [83]. Granzyme A, on the other hand, is completely caspase-independent. Granzyme A cleaves the nucleosome assembly protein SET, which normally inhibits NM23-H1, a DNAse, thereby promoting DNA nicking and ultimately DNA degradation [83,97].

**1.2.3 Dysregulated apoptosis and pathology**

Dysregulation of apoptosis is implicated in a wide variety of diseases. Excessive apoptosis could lead to neurodegenerative diseases and ischemic damage; whereas insufficient apoptosis could give rise to inflammatory diseases, autoimmune disorders and many types of cancer [83].

*Insufficient apoptosis.* Cancer occurs when there is an inadequate level of apoptosis, whether due to over-proliferation of cells and/or decreased removal of cells [83]. Tumor cells
resist apoptosis by expressing anti-apoptotic proteins (e.g. Bcl-2) or by down-regulating or mutating pro-apoptotic proteins (e.g. Bax) [83]. They can also evade killing by immune cells by down-regulating their Fas receptors [83], expressing non-functioning Fas receptors [98], or secreting soluble Fas receptors to sequester the Fas ligands [99]. Aside from cancer, another disease afflicted by insufficient apoptosis is autoimmune lymphoproliferative syndrome (ALPS) [100], which is a collection of diseases that include hemolytic anemia, immune-mediated thrombocytopenia, and autoimmune neutropenia [83]. These diseases occur due to insufficient apoptosis of auto-aggressive T-cells, or over-proliferation of B-cells that results in excessive immunoglobulin production, both leading to autoimmunity [83].

Excessive apoptosis. Although caused by the human immunodeficiency virus (HIV), AIDS is an example of illness mediated by excessive apoptosis. The HIV Tat protein is thought to increase Fas receptor expression on the virus-infected CD4+ T-cell, and therefore upon engagement of the death receptors, result in excessive apoptosis via the extrinsic pathway [83]. The loss of CD4+ T-cells in AIDS patients attenuates their immune system and increases vulnerability to other illnesses. The neurodegenerative disease, Alzheimer’s, is thought to be caused by mutations that result in amyloid β protein to be deposited extracellularly in aggregated plaques. Amyloid β induces excessive apoptosis by causing oxidative stress or by triggering increased FasL expressions in neurons and glia [83].

1.2.4 Role of apoptosis in TB pathogenesis

Apoptosis is an important cellular process that contributes to host defence strategies against intracellular infections, including viral, fungal, and bacterial pathogens [90]. In fact, virulent Mtb strains actively block host macrophage apoptosis to persist and replicate
intracellularly [101]. Conversely, increased apoptosis is observed in macrophages infected with avirulent Mtb strains [101]. Inhibition of host cell apoptosis has two benefits to the pathogen: (1) it preserves the protective niche for intracellular growth, and (2) reduces priming of adaptive immunity by limiting exposure of antigens. Whereas, host cell apoptosis removes the protective niche that harbours intracellular pathogens and also it encases bacterial antigens in apoptotic bodies [102]. These membrane-bound vesicles display “eat me” signals for non-infected bystander professional phagocytes to take up (efferocytosis), and thereafter, present to and activate lymphocytes [90]. This process thus forces the pathogen to have to re-establish itself in a new naïve host cell.

Therefore, the outcome of TB infection in an individual depends on who wins the tug of war between establishing or inhibiting apoptosis, since in the early stages of infection, Mtb adopts a primarily intracellular lifestyle. Virulent Mtb evolved various strategies to block the host cell apoptosis mechanisms. For example, it has been found that the nuoG and secA2 genes in Mtb inhibit apoptosis. NuoG encodes a subunit of the multicomplex component type I NADH-dehydrogenase [103]. In a gain-of-function experiment, introduction of nuoG into non-pathogenic mycobacteria gave rise to the ability to inhibit apoptosis in human or mouse macrophages, and increased virulence in SCID mice [103]. Conversely, deletion of nuoG in Mtb led to more apoptosis and significantly reduced its virulence in mice [103]. SecA2 encodes a component of a virulence-associated protein secretion system in Mtb [64]. Mutations in the secA2 gene impairs secretion of superoxide dismutase and converts virulent Mtb (H37Rv) into a pro-apoptotic strain [64]. Mice infected with secA2 mutant therefore has increased priming of MHC I (CD8+*) immunity [64] and vaccination of mice and guinea pigs with this strain induced protective immunity superior to BCG [64].
Virulent Mtb has also been found to inhibit apoptosis by interfering with TNFα signaling and by upregulating expression of anti-apoptotic protein Mcl-1 [104-106]. Mtb can block TNFα-mediated apoptosis [90] by inducing macrophage secretion of TNFR2 to sequester TNFα [107]. Fas ligand expression is also down-regulated in Mtb-infected macrophages [90].

1.2.5 Role of apoptosis in TB immunity

While efferocytosis of Mtb-infected apoptotic bodies by macrophage lightens the bacillary load, perhaps more importantly, is the efferocytosis by dendritic cells (DC). DC are professional APCs that are of utmost importance in mounting an adaptive immune response. Schaible et al. [55] isolated macrophage apoptotic bodies containing BCG, fed them to immature DC, and found that these DC efficiently activated antigen-specific T-cells including MHC I-restricted CD8+ T-cells. This suggests that macrophage apoptosis is beneficial to the host by promoting priming of adaptive immunity, and in particular by facilitating cross-priming of Mtb antigen to activate CTL [90]. Molloy et al. [108] reported that inducing apoptosis in BCG-infected macrophages using an exogenous drug decreased bacillary viability. The reverse is observed when macrophages were encouraged to undergo necrosis. Similarly, Fratazzi et al. [109] showed that if fresh, uninfected macrophages were added to cultures of *M. avium* (an avirulent, pro-apoptotic mycobacteria strain)-infected macrophages that were undergoing apoptosis, the result is also reduced bacterial viability. However, this phenomenon is not observed if the infected cells were undergoing necrosis [109]. More importantly, the killing of *M. avium* appears to be contact-dependent, suggesting that it is likely due to engulfment of apoptotic bodies of infected macrophages by naïve macrophages [109]. The same effect is observed in Mtb infections. Lee et al. [108] showed
that adding fresh macrophages to Mtb-infected macrophages undergoing apoptosis caused reduction to Mtb viability. Conversely, if macrophages were added to these cells when they were left to undergo necrosis (at a much later time point), Mtb growth was observed. This is likely due to the efferocytosis of apoptotic bodies from infected macrophages harboring Mtb overcoming the typical resistance to phagolysosomal fusion and delivering the pathogen to an acidified phagolysosome [90].

1.3 Caspase-3

1.3.1 Caspase-3 processing and activation

Caspases are classified as cysteine aspartate proteases, widely expressed in most cells in the inactive, proenzyme form [83]. Once activated, they can activate other pro-caspases via proteolytic cleavage, or can aggregate and auto-activate [83]. Both mechanisms result in a protease cascade that amplifies the apoptotic signaling pathway and lead to rapid cell death [83]. Caspases exert their proteolytic activity at aspartic residues, using specificity that is attributed by neighboring amino acids [83]. Ten major caspases have been identified to date, and are categorized into two groups for apoptosis: (1) initiators (caspase-2, -8, -9, -10), and (2) effectors or executioners (caspase-3, -6, -7) [108,110].

Pro-caspase-3 becomes activated when two cleaved monomers come together to form an active dimer [111]. Active caspase-3 recognizes a short cleavage motif (DXXD) and cleaves proteins containing this motif [112,113]. Although caspase-7 recognizes the same motif towards synthetic substrates, it is a functionally distinct protease from caspase-3, which is still regarded as the more important player in the final phase of apoptosis due to its substrate promiscuity [114,115]. Once caspase-3 is activated, there is no turning back from
programmed cell death. Therefore, the activity of caspase-3 is tightly controlled by a constant turnover rate, which ensures the threshold of this protease would not be reached unless an apoptosis stimulus is present [116]. In fact, in eukaryote cells, a sub-apoptotic level of caspase-3 is present independent of apoptosis. Besides merely being a cell death effector, caspase-3 can participate in fundamental cell processes such as proliferation, migration, and differentiation [117,118].

1.3.2 Execution of apoptosis by caspase-3

Caspase-3, -6, and -7 activate cytoplasmic endonucleases, which degrade nuclear materials [83]. They also activate other proteases that degrade nuclear and cytoskeletal proteins [83]. However, caspase-3 is regarded as the most important executioner caspase. It specifically activates the endonuclease CAD (caspase-activated DNase) by cleaving ICAD (the inhibitor of CAD). Cleaved ICAD releases CAD [119], which then is free to degrade chromosomal DNA in the nuclei and causes chromatin condensation [83]. Caspase-3 also causes cytoskeletal reorganization by cleavage of the actin binding protein gelsolin [83]. This causes cell disintegration into apoptotic bodies.

The ultimate goal of apoptosis is for apoptotic bodies to be cleaned up by nearby phagocytic cells. Caspase-3 is found to be one of the molecules involved in the regulation of phosphatidylserine (PS) externalization on oxidatively stressed erythrocytes [120]; although, caspase-independent PS exposure occurs in apoptotic primary T-lymphocytes [121]. Exposure of PS on apoptotic cells facilitate non-inflammatory phagocytic recognition by macrophages and fibroblasts and contributes to immune clean-ups [122].
1.4 Cathepsin S

1.4.1 Overview of cysteine cathepsins

Cysteine cathepsins are members of the papain-like cysteine proteases family [123]. They are widely distributed among living organisms, and have an uneven tissue-specific expression pattern [123]. There are 11 human cysteine cathepsins discovered based on different DNA sequences – Cathepsin B, C, F, H, K, L, O, S, V, X and W [124]. Cysteine cathepsins generally reside in the lysosome and require a reducing, slightly acidic environment for optimal activity [123]. The majority of cathepsins are ubiquitously expressed in human tissue, indicating that they function in normal cellular protein degradation and turnover; whereas cathepsins K, W, and S have a restricted cell- or tissue-specific distribution, therefore likely to have more specific roles. For example, cathepsin S (CatS) is predominately expressed in APCs, such as macrophages, DC and B cells [125].

Initially, cathepsins were thought to only reside and be active in lysosomes, therefore they were considered to be strict intracellular enzymes responsible for non-specific, bulk proteolysis in the endosomal/lysosomal compartments, where they simply degrade intra- and extracellular proteins [123]. It was later found that active cathepsins are also located in other cellular compartments, such as the nucleus, cytoplasm and plasma membrane [123]. For example, it has been shown that nuclear cathepsin L (CatL) plays a role in cell-cycle progression by proteolytic processing of histones [126,127]. Most cysteine cathepsins function best in slightly acidic pH and are usually unstable at neutral pH, as they will rapidly become irreversibly inactivated [123,128]. However, CatS is an exception, where it is stable at neutral or even slightly alkaline pH [129].
Lysosomal cathepsins are synthesized as pre-proenzymes [123]. It is comprised of an N’ terminal signal peptide, which gets cleaved during passage to the endoplasmic reticulum (ER) [123]. The pro-domain (propeptide) assists in the proper folding of the enzyme and targets it to the endosome/lysosome using a specific mannose-6-phosphate receptor (M6PR) pathway [123]. The pro-domain is also an inhibitor to its cognate cathepsin [123]. After removal of the pro-domain, the cathepsin is fully mature and active. Activation occurs at acidic pH and the propeptide dissociates from the enzyme surface [123].

Cysteine cathepsins exhibit broad specificity, cleaving their substrates preferentially after basic or hydrophobic residues [123]. Originally cysteine cathepsins were thought to only participate in terminal protein degradation during cell death, however, it is now well recognized that they contribute to a variety of physiological functions. For example, they take part in MHC II-mediated antigen presentation, bone remodeling, keratinocyte differentiation, and prohormone activations [123]. Altered cathepsin expression and activity levels could lead to various pathological conditions, such as neurological disorders, cardiovascular disease, obesity, inflammatory disease, and cancer [123]. Interestingly, polymorphism in the CatX gene was found to be associated with susceptibility to TB, possibly related to its hypothesized role in immune functions [130].

1.4.2 Overview of MHC presentation pathway

The process of antigen presentation requires T-lymphocyte to recognize antigens presented in the form of short peptides bound to a major histocompatibility complex (MHC) molecule on the surface of APC [131]. Depending on the origin of the antigen, the peptides would either be presented on MHC I or MHC II molecules. When the antigen is generated in
the cytosol, namely from intracellular pathogens or viral infections, the antigen is presented on MHC I. When the antigen is generated from the endosomal/lysosomal compartment, mostly due to endocytosis or phagocytosis by the APC, the antigen is presented on MHC II, to be recognized by CD4+ T-cells, whereas MHC I-antigen complex is detected by CD8+ T-cells.

The MHC I antigen presentation pathway begins with proteins being processed by the proteasome, using subunits encoded by the low molecular weight polypeptide (LMP) genes in the cytosol [132]. The resulting peptides are then translocated into the ER lumen via the transporter associated with antigen processing (TAP) [132]. In the ER, TAP has an additional role in peptide loading onto the MHC I heterodimers [132]. Since LMPs and TAPs are instrumental in the processing and loading of peptides onto MHC I, naturally, variability in these two molecules could lead to differential susceptibility to pathogens. Indeed, polymorphisms in the LMP and TAP genes are found to be associated with increased susceptibility to TB in a subpopulation in China [133].

Before the antigen can be loaded onto MHC II molecules, the MHC must first undergo a highly regulated maturation process. MHC II are assembled in the ER into a trimer, comprised of the alpha and beta heterodimer associated with the invariant (Ii) chain (Fig. 3) [134]. The Ii is a chaperone molecule that is a type II glycoprotein that promotes the proper folding and assembly of MHC alpha/beta heterodimer [135,136]. The alpha/beta heterodimer binds to Ii at a particular region called CLIP (class II-associated Ii peptide) in its peptide-binding groove [137]. Ii stabilizes the nascent MHC II heterodimer and prevents premature binding of polypeptides or partially folded proteins in ER [137]. Ii has an endosomal/lysosomal targeting motif at the cytoplasmic tail, to traffic the MHC II to
endosomes [138,139]. In order for the antigen to be mounted into the peptide-loading groove, the Ii chain must be degraded by lysosomal proteases in a step-wise fashion (Fig. 4). This degradation occurs in the late endosome/pre-lysosome stage [134]. When Ii is degraded into CLIP, the MHC II-like chaperone molecule HLA-DM exchanges CLIP with peptides derived from self or foreign protein antigens (Fig. 5). The MHC II-peptide complex then traffics to plasma membrane.

Figure 3. Model of the MHC alpha/beta hetero-dimer associated with Ii chain. The alpha/beta heterodimer is represented by the blue and purple colors. The Ii chain is represented by green color, where the red section is the CLIP peptide. Cresswell P. Cell. 1996. 84(4):505-7. Reprinted with permission from Elsevier.
Figure 4. Invariant chain degradation. The Ii chain is degraded in a step-fashion by multiple proteases before finally taking the form of CLIP peptide. Rudensky A and Beers C. Ernst Schering Res Found Workshop. 2006. (56):81-95. Reprinted with permission from Springer.
1.4.3 Role of CatS in MHC II maturation

Both CatS and CatL are required for late stage Ii processing in professional APCs, but in distinct cell types. They were initially thought to perform similar functions, however, it
was later discovered that they indeed have specific non-redundant roles. CatS and CatL are differentially expressed in APCs, including B cells, macrophages, DC, specialized thymic and intestinal epithelium cells [137]. CatS activity was found in B cells and DCs, while CatL activity was found in cortical thymic epithelial cells, whereas macrophages express both CatS and L [137].

Their non-redundant function was elucidated by CatS-deficient mouse experiments that showed B cells and DC contain MHC II-associated Ii degradation intermediates halted at p12 and p18 fragments [140,141]. Antigen presentation studies using MHC II-restricted T-cell hybridomas showed that they have diminished presentation of the majority of exogenous antigens tested when compared to wild-type mice. In CatL-deficient mice, however, B cells and DCs showed normal Ii degradation and MHC II antigen processing and presentation. In contrast, defect in CatL presented a substantial amount of MHC II on cortical thymic epithelial cells (cTECs) bound to p12 Ii fragments. Thus, CatL is important in Ii degradation in the thymic epithelium and purified cTECs [142,143], affecting positive selection of immature double-positive thymocytes.

In macrophages, CatS is the predominant enzyme that processes Ii, as CatL’s role in IFNγ stimulated macrophages are not detected [144]. In fact, it was found that CatL activity is down-regulated upon IFNγ stimulation by CatL-specific inhibitors [137]. Similar phenomenon has been observed in DC. This suggests that in a Th1-mediated immune response, which is dominated by IFNγ production, CatS is preferentially used in all bone marrow derived APCs in secondary lymphoid organs [137]. CatS is crucial in bone marrow-derived cells that induce immune response in the periphery [137], such as DC and
macrophages [123]. However, it is uncertain whether CatS simply degrades the Ii into smaller peptides or it performs a specific cleavage [123].

1.4.4 Lysosomal pathway of apoptosis

Lysosomes and their proteases have always been associated with cell death. However, while their high potential of degradation was known to be involved in autophagy and necrosis, little was known about their role in apoptosis until the 1990s [145]. The first cathepsin to be linked to apoptosis was CatD, which was once thought to be the lone cathepsin among the lysosomal proteases [146]. We now know that cathepsins are important players in apoptotic pathways; they may act on and activate cell death effector proteases such as granzymes, or directly act as cell death effectors [146].

During lysosome-mediated apoptosis, a critical step is the destabilization of the lysosomal membrane, causing the release of cathepsins into the cytosol. Lysosomal membrane can be disturbed by lysosomotropic agents such as L-leucyl-L-leucine methyl ester (LLOMe) [147], N-dodecylimidazole (NDI) [148], sphingosine [149], and the quinolone antibiotics ciprofloxacin and norfloxacin [150]. In terms of endogenous stimuli for lysosomal membrane permeabilization, sphingomyelin and reactive oxygen species (ROS) can destabilize the membrane [146]. Sphingomyelin can be converted to ceramide, and further to sphingosine. The accumulation of ceramide or sphingosine increases the permeability of the lysosomal membranes [146]. Ceramide can also specifically activate CatD [151] and lead to apoptosis in this manner. ROS destabilizes the lysosomal membranes by the peroxidation of membrane lipids [146]. Superoxide radicals ($O_2^-$) are further reduced to hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD), and $H_2O_2$ is lysosome
permeable [146]. In the lysosome, the acidic pH and high iron content encourages a Fenton-type reaction and generates intralysosomal ROS, in particular the hydroxyl radical HO'. Since mitochondria are major generator of ROS and H₂O₂, these agents act as cross-talk between organelles. Furthermore, the signals could be amplified in lysosome through Fenton reactions of converting H₂O₂ to hydroxyl radicals.

Unlike other cell types, CTLs do not possess conventional lysosomes, but their lytic granules serve as a lysosome-related organelle [152]. These granules contain perforin and granzymes renowned for CTL’s cytotoxicity. Perforin is synthesized as an inactive molecule and becomes active after proteolytic removal of the C-terminal peptide [153], most likely by cysteine cathepsins [153]. Granzymes are also synthesized as inactive zymogens and their activation require N-terminal dipeptide cleavage, mediated by CatC residing in the lytic granules [146]. In the case of granzyme B, activation is performed by CatH, and possibly by other proteases [154].

Unlike caspases, cathepsins are already active once exported from the lysosome into the cytosol [155]. The first and best-established cathepsin substrate is Bid [156-160], which was initially described as a caspase-8 substrate. Bid was found to be targeted by many proteases, as the activation site is located in a large unstructured loop called the bait loop [146]. It can be cleaved by caspase-8, granzyme B, calpains, and most of the cathepsins [146,156]. In cell-free systems, Bid was found to be cleaved by CatB, K, L, and H [157], and these reactions were confirmed in various cellular models [157,158]. However, Bid cleavage is not the only way cathepsins regulate apoptosis since cathepsin-mediated apoptosis still occurs in Bid-deficient mice [161]. Indeed, other cathepsin substrates were found to include the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-XL, Mcl-1 and Bax [123,158,159].
of them can be cleaved by multiple cathepsins except Bax, which was found to be cleaved only by CatD [158,162]. All of the above suggests that cathepsins act upstream of mitochondrial membrane permeabilization events, and executes apoptosis using the mitochondrial pathway [123]. However, X-chromosome-linked inhibitor of apoptosis (XIAP) is also degraded by cysteine cathepsins [158], suggesting that cathepsins can also act downstream of mitochondrial pathway and perhaps target other apoptosis inhibitors [146]. Furthermore, the finding that caspase-8 is directly activated by CatD during neutrophil apoptosis suggest that cathepsins may have the ability to act on caspases directly [163].

Due to extreme sensitivity of CTLs to the lysosomotropic agent LLOMe, the chemical was proposed as a therapeutic drug in transplant surgery since it targets the lytic granules and induces apoptosis in CTLs during allogenic bone marrow transplantation (currently in clinical trial) [146]. LLOMe can also be used for ex-vivo removal of perforin-positive T-cells from donor lymphocytes used to regenerate immune competence in patients receiving allogeneic bone marrow transplantation [146].

1.5 Aim of study

The overall objective of this thesis is to engineer a highly immunogenic strain of BCG vaccine by making it more apoptosis-inducing via the introduction of genes that encode apoptosis-mediating factors.

BCG prevents host cell apoptosis even in the presence of potent apoptosis-stimulating agents. Preliminary work demonstrated that CatS exerts numerous physiological functions. Instead of a sole responsibility in the canonical function of invariant chain processing to mature MHC II molecules, CatS is likely to be also involved in the apoptosis pathway. Thus,
we decided to construct a recombinant BCG stably expressing CatS (rBCG-CatS) and used it to check whether this strain induces more apoptosis in infected host cells, and as a result achieve a greater immune response in the murine model when compared to BCG. Based upon these findings, and considering that macrophage apoptosis drives optimal adaptive immunity, we went one step further and generated a recombinant BCG strain expressing caspase-3, which is the major apoptosis executioner in cells.

Therefore, the working hypothesis of the thesis is: a pro-apoptotic recombinant BCG will induce host cell apoptosis and therefore elicit greater immune response in the murine model.

Overall, this thesis has provided significant insight into creating a highly immunogenic BCG vaccine via promoting macrophage apoptosis. We have shown that macrophage apoptosis connects one of the missing links between innate and adaptive immunity, since apoptosis overcomes the antigen presentation hurdle that BCG vaccination imposes. By demonstrating that a pro-apoptotic BCG improves immunogenicity, this work helps pave the way for a more efficacious TB vaccine to prevent this deadly disease.
CHAPTER 2: MATERIALS AND METHODS

2.1 Reagents and chemicals

Cell culture media (RPMI 1640 and DMEM) and miscellaneous culture reagents were purchased from StemCell Technologies (Vancouver, BC, Canada). Fetal calf serum (FCS) was purchased from Gibco Laboratories (Burlington, ON, Canada). PMA, mammalian protease inhibitor, and PMSF were purchased from Sigma-Aldrich (St. Louis, MO). Oleic acid albumin dextrose catalase complex (OADC), 7H9 and 7H10 agar culture media were from Difco Laboratories (Detroit, MI). Chloramphenicol was purchased from Sigma-Aldrich and kanamycin, hygromycin and zeocin were from Invitrogen (Burlington, ON, Canada). IL-2 ELISA standard and antibodies were purchased from Peprotech (Quebec, ON, Canada). Calf intestine alkaline phosphatase (CIAP) was purchased from Fermentas (Burlington, ON, Canada). BP and LR clonases, reading frame cassettes (attR1-cm-ccdB-attR2), and ccdB Survival T1 E. coli were from Invitrogen. Luria–Bertani (LB) broth and LB agar were from Fisher Scientific (Pittsburgh, PA). Plasmid purification Miniprep kit was from Qiagen (Mississauga, ON, Canada). Staurosporine was purchased from Sigma-Aldrich and were used at 100 nM. Four μm surfactant-free white sulfonate latex beads were purchased from Interfacial Dynamics Corp (Portland, OR). Phycoerythrin (PE)-conjugated I-A^b-P25 (Ag85B:240-254) tetramers were obtained from NIH Tetramer Core Facility (Atlanta, GA). CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit and Alexa Fluor (AF) 488 conjugated annexin V were purchased from Invitrogen.

2.2 Antibodies

Rabbit polyclonal anti-human CatS antibody was purchased from Millipore (Billerica, MA). Caspase-3, cleaved caspase-3, and PARP antibodies were purchased from Cell
Signaling (Danvers, MA). Rabbit anti-actin antibody was purchased from Santa Cruz. Dylight 680-conjugated goat anti-rabbit IgG (H+L) antibody was purchased from Thermo Fischer Scientific (Burlington, ON, Canada). and AF647 goat anti-rabbit were purchased from Invitrogen. AF647 rat anti-mouse CD4, AF647 rat anti-mouse CD8, PE-Cy7 rat anti-mouse CD4, PE rat anti-mouse CD8, AF647 rat anti-mouse IFNγ, and AF647 rat anti-mouse TNFα antibodies were purchased from BD Bioscience (Mississauga, ON, Canada). AF647 rat anti-mouse IL-2 antibody was from Biolegend (San Diego, CA).

2.3 Cell culture

2.3.1 Cell line maintenance and propagation

RAW 264.7 macrophages (American Type Culture Collection, Manassas, VA) were maintained at 37°C and 5% CO₂ using 10 cm diameter culture dishes (Corning Inc., Corning, NY) at a density of ~10⁵ per cm² in DMEM supplemented with 10% FCS and 1% each of L-glutamine, penicillin and streptomycin, HEPES, non-essential amino acids (100x solution, StemCell). The pro-monocytic THP-1 cell line (American Type Culture Collection) was PMA-differentiated into adherent macrophage-like cells as described [164].

2.3.2 BMDM culturing

BMDM (bone marrow derived macrophages) were harvested from femurs of female C57BL/6 mice (I-Aᵇ, H-2Kᵇ, 5-6 week old). Mice were obtained from Charles River Laboratories (Sherbrooke, QC, Canada) and were housed under specific pathogen-free conditions in the animal biosafety level II facilities of the Jack Bell Research Centre (Vancouver, BC, Canada). Bone marrow derived cells were cultured and differentiated into macrophages using complete RPMI media supplemented with 10% L-cell Conditioned
Media (LCM). BMDM maturation occurs at day 7 post-harvest and cells were detached from culture dishes using (non-enzymatic) cell dissociation solution from Sigma-Aldrich.

2.3.3 Infection

Macrophage monolayers at the appropriate cell density (2 x 10^5 per cm^2) were exposed to bacteria or beads at listed multiplicity of infections (MOIs) for 3-4 h at 37°C to allow for full internalization and then non-ingested particles were removed by washing extensively with culture media. Unless otherwise specified, infected cells were re-incubated for overnight in fresh culture media.

2.4 Bacteria

2.4.1 Mycobacterial strains and growth conditions

*M. bovis* BCG (Pasteur 1173P2) was obtained from Dr. Richard Stokes (Department of Microbiology and Immunology, University of British Columbia). BCG expressing GFP was generated as described in Sun et al. [165]. rBCG-CatS and –C3 was obtained by electroporation of competent bacteria with integrative plasmid pJAK1.A-CatS or pJAK1.A-C3 as described in section 2.4.2, respectively. Parental and recombinant BCG strains were grown in Middlebrook 7H9 broth supplemented with 10% (v/v) OADC, 0.2% (v/v) glycerol and 0.05% (v/v) Tween 80 (Sigma-Aldrich) at 37°C on a shaker platform at 50 rpm, and appropriate antibiotics were added where applicable.

2.4.2 Construction of mycobacterial Destination Vectors

During my pre-doctoral training in Dr. Hmama’s laboratory, I generated the following recombination-compatible, mycobacterial vectors that have proved to be essential tools to my PhD research.
This work was published in:


Reprinted with modifications with permission from Elsevier.

pMV261 and pMV361 vectors were linearized by the blunt cut restriction enzyme PvuII within their multicloning sites. The vectors were then treated with CIAP for 30 min at 37°C, followed by inactivation at 85°C for 15 min to dephosphorylate the 5’ end in order to prevent self-ligation. The linearized vectors were then ligated with the appropriate blunt reading frame cassette (attR1–cm–ccdB–attR2) with T4 DNA ligase overnight at 16°C. The ligated product was then transformed into ccdB Survival T1 E. coli, and positive clones in the correct orientation of the cassette were screened by PCR using specific primers.

Recombination-compatible Destination Vectors pMV261 and pMV361 were renamed to be pJAK2.A and pJAK1.A, respectively.

BP reactions were performed for cloning of PCR products (GOI) into Entry Vectors, which is necessary for the eventual cloning of the segment into Destination Vectors. The attP entry plasmid pDONR221/Zeo (150 ng) was mixed with 1–3 µL of each purified PCR product in reactions (10 µL) that contained 2 µL BP Clonase in 25 mM Tris–HCl, pH 7.5, 22 mM NaCl, 5 mM EDTA, 5 mM spermidine HCl, 1 mg/mL BSA. After 2 h incubation at 25°C, proteinase K (2 µg in 1 µL) was added, and each reaction was incubated at 37°C for 10 min. Aliquots (2 µL) of each reaction were transformed into E. coli TOP10 and plated on zeocin LB plates (50 µg/mL). Positive clones were screened by colony PCR and the subsequent mini-prepped DNA were subjected to Sanger sequencing.
The GOI in positive Entry Vector clones utilizes the LR reaction for transference into mycobacterial Destination Vectors. Aliquots containing 150 ng of each miniprep DNA of Entry clones were incubated with 150 ng of the appropriate Destination Vector in 10 μL reactions containing 2 μL of LR Clonase, 50 mM of Tris-HCl, pH 7.5, 50 mM of NaCl, 0.25 mM of EDTA, 2.5 mM of spermidine HCl, and 0.2 mg/mL of BSA. Then proteinase K (2 μg in 1 μL) was added, and reactions were incubated at 37°C for 10 min. Aliquots (2 μL) of each reaction were transformed into E. coli TOP10 and plated on kanamycin (50 μg/mL) LB plates. Positive clones were screened by colony PCR and Sanger sequencing.

2.4.3 rBCG-CatS and rBCG-C3 constructions

pJAK1.A-CatS (Fig. 7A) was constructed as follows: the active site domain of human CatS (position 115-331 according to Uniprot accession #P25774) was synthesized by GenScript (Piscataway, NJ) to be flanked with the E. coli hemolysin A nucleotide sequence (hlyA) on both sides. In addition, the mycobacterial Ag85B (fbpB) signal sequence and the first 33 nucleotide base pairs of its mature protein is placed upstream of the hlyA sequence, where downstream of the hlyA is completed with extra spacer sequences. The synthesized gene flanked with attB1 and attB2 adapters was cloned in entry vector pDONR221/Zeo, then in destination vector in frame with the Hsp 60 promoter using our Gateway-compatible vector pJAK1.A as described in Sun et al. [165] and Section 2.4.2.

pJAK1.A-C3 (Fig. 14A) was constructed by replacing the CatS segment in pJAK1.A-CatS, with a synthesized DNA segment encoding for reverse (active) caspase-3 as described in Srinivasula et al. [166].
2.4.4 Mycobacteria transformation

Competent BCG (400 μL) were mixed with 1 μg of DNA and transferred to an electroporation cuvette of 0.2 cm diameter (Bio-Rad, Hercules, CA). Bacteria were electroporated with 2.5 V, and allowed to recover in 7H9 supplemented with 10% OADC in the absence of antibiotics for overnight. Bacteria were then plated on 7H10–OADC in the presence of appropriate antibiotic.

2.4.5 Mycobacteria expression

rBCG-CatS and –C3 was verified for CatS or caspase-3 expression, respectively, by growing the bacterial culture to mid-log phase. Cultures were normalized using optical density at 600 nm and ~10⁹ bacteria were used per strain. After washing thrice with PBS + 0.05% Tween 80, cell pellets were suspended in 62.5 mM Tris at pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% beta-mercaptoethanol and bromophenol blue at 65°C for 30 min to recover bacterial surface proteins. Cells were pelleted again and supernatants were boiled and resolved on a 15% SDS-PAGE gel. Proteins were then transferred onto nitrocellulose membrane using the semi-dry method at constant 200 mAmp for 35 min. Membranes were blocked using TBS-T + 3% skim milk powder and probed with anti-CatS or anti-caspase-3 antibody at 1:1000 for overnight at 4°C. Blot was developed on the Odyssey Clx from Li-Cor (Lincoln, NE) using goat anti-rabbit IgG (H+L) conjugated with DyLight 680.

2.5 Microarray and RT-qPCR study

PMA-differentiated THP-1 cells were left untreated (control) or infected at MOI 20 with BCG or BCG transformed with an episomal plasmid pSMT3 expressing human CatS (rBCG-hcs) [167]. Total RNA was isolated at 6 h post-infection, then 10 μg of each RNA
sample were first reverse transcribed into cDNA prior to hybridization with the cDNA derived from an universal RNA (Stratagene, La Jolla, CA) and hybridized on Operon’s Human Genome OpArray™ version 4 slides. cDNA indirect labelling was carried out using Genisphere’s Array 350 Expression Array Detection Kit (Genisphere Inc. Hatfield, PA). Analysis of the DNA microarray data was performed with the GenePix Autoloader 200AL (Molecular Devices, Sunnyvale, CA), ImaGene (BioDiscovery, El Segundo, CA) and GeneSpring GX (Agilent Technologies, Santa Clara, CA) softwares.

RAW 264.7 cells were infected with either BCG or rBCG-CatS at MOI 20 for 6 h prior to RNA isolation using RNeasy Mini Kit from Qiagen (Mississauga, ON, Canada). cDNA synthesis was performed using EasyScript cDNA Synthesis Kit from ABM Inc (Richmond, BC, Canada). RT-qPCR was performed using gene-specific primers listed in Table 1, with EvaGreen qPCR MasterMix from ABM Inc. The reactions were performed on the StepOnePlus system (Thermo Fisher Scientific, Burlington, ON, Canada), using standard qPCR reaction parameters (enzyme activation: 95°C, 10 min; denaturation: 95°C, 15 sec; annealing/extension: 60°C, 60 sec).
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer set</th>
</tr>
</thead>
</table>
| AATF        | Fw: 5’ CCAAGGGTGATTGACAGGT'TTG 3’  
              |             |
|             | Ry: 5’ CCAAGTTTCTAATGTGCTACCCACT 3’ |
| BAD         | Fw: 5’ CCCAGAGTTTGAGCCGAGTG 3’  
              |             |
|             | Ry: 5’ CCCATCCTTTCTGTCGTCCT 3’ |
| Bcl-2       | Fw: 5’ GGTGGGGGTCTGATGCTGTG 3’  
              |             |
|             | Ry: 5’ CGGTCAGGTCACGTCCCAT 3’ |
| DAP         | Fw: 5’ AATGCGAATTGTCAGAAACAC 3’  
              |             |
|             | Ry: 5’ GGGCTTTCCATTTCTGGTC 3’ |
| Mcl-1       | Fw: 5’ GTGCCCTTTGTGGCTAACACT 3’  
              |             |
|             | Ry: 5’ AGTCCCCGTATTGTCCACGGTA 3’ |
| Puma        | Fw: 5’ GACCTCAACGCACATGACAGAG 3’  
              |             |
|             | Ry: 5’ AGGAGTCCCATGAGATTGT 3’ |

Table 1. List of primers used in RT-qPCR study.

2.6 Apoptosis analysis

2.6.1 Annexin V microscopy

To perform annexin V staining, adherent cells on cover slips were washed twice in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl$_2$ at pH 7.5) and surface stained with AF488-annexin V at 1:20 for 30 min. Cells were then washed in binding buffer and cover slips were mounted on microscope slides in ProLong Gold anti-fade plus DAPI (Thermo Fisher Scientific) to minimize photo-bleaching. Thereafter, slides were examined by digital confocal microscopy using an Axioplan II epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY) equipped with 10x Plan-Apochromat objective (Carl Zeiss Inc.). Images were recorded using a CCD digital camera (Retiga EX, Q Imaging, Burnaby, BC, Canada) coupled to the AxioVision software (Carl Zeiss Inc.).
2.6.2 Flow cytometry

Levels of cleaved caspase-3 were measured by a flow cytometry assay. Adherent macrophages were lifted from the culture plate, fixed with 4% PFA and permeabilized with 0.2% Triton X-100 in 1x PBS buffer. Cells were then blocked with 0.1% Triton X-100 and 2% BSA in 1x PBS and stained for 1 h with cleaved caspase-3 antibody (1:250) in FACS buffer (2% FCS in 1x PBS) then with AF647 goat anti-rabbit antibody at 1:1000 for 30 min. Cells so treated were washed and suspended in FACS buffer then analyzed with a BD FACSCalibur flow cytometer.

2.6.3 Western blot analysis

Cells were washed thrice with cold 1x PBS after infection and lysed using cell extraction buffer (10 mM Tris at pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₃P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1% mammalian protease inhibitor and 1 mM PMSF). Cell lysate concentrations were determined and normalized using DC protein assay from Bio-Rad (Hercules, CA). Lysates were resolved and transferred into membranes as described above. For caspase-3, membranes were blocked using TBS-T + 5% milk and probed with caspase-3 and actin antibodies at 1:1000 for overnight. For PARP, membranes were blocked using TBS-T + 2% BSA and probed with PARP antibody at 1:1000 for overnight. Blots were developed on the Odyssey Clx from Li-Cor using DyLight 680-conjugated goat anti-rabbit IgG.
2.7 Murine immunogenicity studies

2.7.1 Mouse immunization and splenocyte harvesting

Four to six weeks old C57BL/6 female mice were obtained from Charles River Laboratories and housed under specific pathogen-free conditions in the animal biosafety level II facilities of the Jack Bell Research Centre. All animals were handled in compliance to protocols approved by the Animal Care and Use Committees at the University of British Columbia. Mice were immunized subcutaneously in the neck scruff with $10^6$ BCG strains in 100 μL PBS. 3 mice were used per treatment group. Bacteria were passed 10 times through 27G½ needles to obtain single cell suspensions prior to immunization. Mice were sacrificed 3-4 weeks post-infection by CO₂ asphyxiation and spleens were excised. Single spleen cells were obtained by crushing the spleens between 2 frosted glasses and passed through 70 μm cell strainers into RPMI media supplemented with 50 μM beta-mercaptoethanol. Red blood cells were depleted using EasySep mouse biotin positive selection kit using biotin-Ter119/Erythroid cells antibody (StemCell). Splenocytes were cultured in RPMI supplemented with 10% FCS and 1% each of L-glutamine, penicillin and streptomycin, HEPES, non-essential amino acids (100x solution, StemCell), and 50 μM beta-mercaptoethanol.

2.7.2 Tetramer staining

Splenocyte samples (4 x $10^7$) were stained with PE-conjugated I-A^b-P25 (Ag85B_{240-254}) tetramers (1:25 in FACS buffer) for 3 h at 37°C, then washed and stained with AF647-conjugated anti-CD4 antibody at 1:50 for 30 min at RT. Cells were washed and fixed with 2% PFA in FACS buffer then analyzed with a BD FACSCalibur flow cytometer. The
frequencies of tetramer positive cells were determined in gated half-million CD4 positive events as described in Liao et al [168].

2.7.3 CFSE cell proliferation assay

Splenocytes (2 x 10^7) were suspended in warm PBS and stained with CFSE at 5 μM for 7.5 min at RT. Cells were then washed thrice with PBS + 10% FCS and suspended in complete RPMI media in the presence of 10 μg/mL BCG whole bacterial lysate (WBL) or 1 μg/mL concavalin A, which is a strong inducer of T-cell division commonly used as positive control. After a 96 h incubation at 37°C, cells were harvested and stained with AF647 conjugated anti-CD4 or anti-CD8 antibody (1:100) for 30 min at RT. Samples were then washed, fixed with 2% PFA then analyzed by flow cytometry. CFSE signal was measured in gated 10^5 positive CD4^+ or CD8^+ events.

2.7.4 Intracellular cytokine staining

Splenocyte samples (3 x 10^7) were stimulated with BCG WBL (10 μg/mL) or 50 ng/mL PMA + 1 μg/mL ionomycin (positive control) and incubated in complete RPMI media for overnight. Thereafter, Brefeldin A (BD Pharmingen) was added at 1:1000 and cells were re-incubated in 37°C for an additional 4 h time period. Cells were then harvested and stained with PE-Cy7 anti-CD4 or PE anti-CD8 antibodies (1:50) at RT for 30 min. Cells were then fixed with 4% PFA and permeabilized with 5% (w/v) saponin in 100 mM HEPES and 10% FCS. Thereafter, cells so treated were divided in 3 aliquots and stained with specific antibodies to IFNγ (1:50), TNFα (1:50), and IL-2 (1:50) for 45 min at RT. Cells were then washed, suspended in FACS buffer and analyzed by flow cytometry. The frequencies of cytokine positive cells were determined in gated 5 x 10^5 positive CD4^+ or CD8^+ events.
2.7.5 IL-2 ELISA

200 µL supernatants were retrieved from the intracellular cytokine staining culture plate (Section 2.7.4) after an overnight incubation with BCG WBL and before the addition of Brefeldin A. Supernatants were spun down and residual cells were discarded. Sandwich ELISA using TMB substrate (BD Bioscience) was performed according to manufacturer’s protocol (Peprotech).

2.8 Statistical analyses

To analyze differences of more than two groups, ordinary one-way ANOVA was utilized. P<0.05 was considered statistically significant and represented by the symbol *. 

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; ns = not significant.
CHAPTER 3: RECOMBINANT BCG EXPRESSING CATHEPSIN S IMPROVES HOST CELL APOPTOSIS AND VACCINE IMMUNOGENICITY

3.1 Background

Apoptosis is an important cellular process that acts as a host defence strategy against intracellular infections, including viral, fungal, and bacterial pathogens [90]. Macrophages infected with mycobacteria that undergo apoptosis, not only remove the protective niche that harbours these intracellular bacteria, but also encase bacterial antigens in apoptotic bodies [102]. These membrane-bound vesicles display “eat me” signals for non-infected bystander dendritic cells (DC) to take up, and thereafter, present to and activate lymphocytes [90]. Thus, it is believed that the inhibitory effect of BCG on host cell apoptosis may hinder its efficacy [169,170].

In order to overcome the limitations of BCG, some investigators focused their efforts towards novel recombinant BCG (rBCG) strains that allow for increased antigen release into the cytosol from its phagosomal confines [71,72]. Others opted for subunit vaccines using viral-vectors expressing immune-dominant antigens dedicated to boost the effect of BCG, also known as the prime-boost strategy [73,74]. Previous work from our laboratory showed that BCG down-regulates mature MHC II surface expression and demonstrated that this phenotype can be reversed by complementing the bacterium with recombinant human CatS [164,167]. While the canonical role of this cysteine protease is its essential involvement in maturation of MHC II molecule via processing of the invariant (Ii) chain [171], like other members in the cathepsin family, CatS may also play a role in apoptosis [158]. Indeed, we demonstrated that in addition to its role in antigen processing, CatS is a key regulator of macrophage apoptosis. We showed that macrophages infected with BCG expressing CatS
(rBCG-CatS) have altered expression of key genes involved in the apoptotic pathway. This effect is more directly illustrated by the introduction of CatS-coated latex beads to the macrophages, which directly induce apoptosis. Most importantly, rBCG-CatS improves immunogenicity in murine model of infection, as indicated by increased antigen-specific T-cell expression and cytokine production. Taken together, rBCG-CatS may hold a novel and promising future in vaccine development in TB research.

3.2 Global macrophage transcriptome profiles in response to BCG expressing Cathepsin S

In a previous study, we showed that BCG inhibits CatS expression in the macrophage [164]. To reverse this phenotype, we used the episomal plasmid pSMT3 to express the active domain of human CatS in BCG and obtained a novel vaccine, rBCG-hcs, capable of delivering active CatS within the macrophage [167]. Unlike parental BCG, rBCG-hcs was capable of inducing phagolysosome fusion and surface expression of mature (antigen-loaded) MHC II molecules in primary and THP-1 derived macrophages [167]. To further characterize rBCG-hcs, we sought to obtain a global picture of cell function changes it might induce in the macrophage. Thus, we applied the DNA microarray technology to examine gene expression changes in infected macrophages relative to untreated cells. The results obtained showed a marked difference in gene expression profiles in rBCG-hcs infected cells compared to BCG-infected macrophages (Figs. 6A and B). In a total of 1328 genes there were 884 differentially expressed genes in response to rBCG-hcs and BCG (Fig. 6C). Of these, 320 genes were up-regulated and 331 were repressed in rBCG-hcs-infected macrophages (Fig. 6C).
Figure 6. Microarray analysis of macrophage transcriptome in response to BCG and rBCG-hcs. PMA-differentiated THP-1 cells were left untreated (control) or infected with BCG or rBCG-hcs. Total RNA was isolated at 6 h post-infection then 10 µg of each RNA sample were first reverse transcribed into cDNA prior to hybridization with the cDNA derived from an universal RNA and hybridized on Operon’s Human Genome OpArray™ version 4 slides. cDNA indirect labelling was carried out using Genisphere’s Array 350 Expression Array Detection Kit. Analysis of the DNA microarray data is performed through the use of GenePix Autoloader 200AL, ImaGene and GeneSpring GX softwares. (A) Represents the tree of hybridization with reds representing increases and green decreases in gene expressions relative to the universal RNA. (B) Represents the scatter plot of normalized intensities of rBCG-hcs against BCG-infected cells. (C) Represents the Venn diagram of differential gene expression between BCG-infected cells and rBCG-hcs-infected THP-1 cells hybridized against control cells. The normalized ratios are divided for each set of comparison.

Upon closer inspection of functional pathway of interest for us, we found that genes associated with phagosome maturation and antigen presentation pathways are significantly up-regulated in cells infected with rBCG-hcs while down-modulated in response to BCG (Table 2), consistent with our previous findings [167]. More interestingly and in contrast to BCG, rBCG-hcs induced the expression of pro-apoptotic genes and repressed those
associated with cell death inhibition. For instance, rBCG-hcs induced 85.37-fold increase in death-associated protein (DAP) while BCG induced 52.43-fold increase in apoptosis antagonizing transcription factor (AATF).

<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>Fold changes between:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCG and Control</td>
</tr>
<tr>
<td></td>
<td>rBCG-hcs and BCG</td>
</tr>
<tr>
<td><strong>Lysosomal pathway</strong></td>
<td></td>
</tr>
<tr>
<td>APG5 autophagy 5-like</td>
<td>-43.478</td>
</tr>
<tr>
<td></td>
<td>114.500</td>
</tr>
<tr>
<td>ATPase, H⁺ transporting, lysosomal, V1 subunit G isoform 2</td>
<td>-1.761</td>
</tr>
<tr>
<td></td>
<td>3.987</td>
</tr>
<tr>
<td>ATPase, H⁺ transporting, lysosomal, V0 subunit D isoform 1</td>
<td>-125.000</td>
</tr>
<tr>
<td></td>
<td>56.900</td>
</tr>
<tr>
<td>ATPase, H⁺ transporting, lysosomal, V0 subunit A isoform 1</td>
<td>-14.706</td>
</tr>
<tr>
<td></td>
<td>88.700</td>
</tr>
<tr>
<td>RAB7B, member RAS oncogene family</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>187.300</td>
</tr>
<tr>
<td>Proteasome (prosome, macropain) 26S subunit</td>
<td>-5.525</td>
</tr>
<tr>
<td></td>
<td>5.206</td>
</tr>
<tr>
<td>Sortilin-related VPS10 domain containing receptor 2</td>
<td>-23.256</td>
</tr>
<tr>
<td></td>
<td>28.900</td>
</tr>
<tr>
<td><strong>Cell death</strong></td>
<td></td>
</tr>
<tr>
<td>Apoptosis antagonizing transcription factor</td>
<td>52.433</td>
</tr>
<tr>
<td></td>
<td>-1.776</td>
</tr>
<tr>
<td>Apoptosis inhibitor 5</td>
<td>1.866</td>
</tr>
<tr>
<td></td>
<td>-2.660</td>
</tr>
<tr>
<td>BCL2-antagonist of cell death</td>
<td>4.238</td>
</tr>
<tr>
<td></td>
<td>-10.101</td>
</tr>
<tr>
<td>BCL2-binding component 3</td>
<td>-1.490</td>
</tr>
<tr>
<td></td>
<td>11.310</td>
</tr>
<tr>
<td>BCL2-like 11 (apoptosis facilitator)</td>
<td>1.551</td>
</tr>
<tr>
<td></td>
<td>6.008</td>
</tr>
<tr>
<td>BCL2-binding component 3</td>
<td>-1.490</td>
</tr>
<tr>
<td></td>
<td>11.310</td>
</tr>
<tr>
<td>BCL2-like 14 (apoptosis facilitator)</td>
<td>-3.175</td>
</tr>
<tr>
<td></td>
<td>4.906</td>
</tr>
<tr>
<td>Caspase 2, apoptosis-related cysteine protease</td>
<td>-11.364</td>
</tr>
<tr>
<td></td>
<td>3.407</td>
</tr>
<tr>
<td>Caspase 3, apoptosis-related cysteine protease</td>
<td>1.968</td>
</tr>
<tr>
<td></td>
<td>10.549</td>
</tr>
<tr>
<td>Caspase 6, apoptosis-related cysteine protease</td>
<td>1.873</td>
</tr>
<tr>
<td></td>
<td>8.140</td>
</tr>
<tr>
<td>Caspase recruitment domain family, member 10</td>
<td>-2.924</td>
</tr>
<tr>
<td></td>
<td>2.571</td>
</tr>
<tr>
<td>Death-associated protein 3</td>
<td>-3.125</td>
</tr>
<tr>
<td></td>
<td>16.417</td>
</tr>
<tr>
<td>Death-associated protein</td>
<td>-1.416</td>
</tr>
<tr>
<td></td>
<td>85.367</td>
</tr>
<tr>
<td>Fas (TNF receptor superfamily, member 6)</td>
<td>-2.212</td>
</tr>
<tr>
<td></td>
<td>6.455</td>
</tr>
<tr>
<td>Proapoptotic caspase adaptor protein</td>
<td>-1.761</td>
</tr>
<tr>
<td></td>
<td>12.076</td>
</tr>
<tr>
<td>Programmed cell death 1 ligand 2</td>
<td>1.299</td>
</tr>
<tr>
<td></td>
<td>2.326</td>
</tr>
<tr>
<td>Programmed cell death 6 interacting protein</td>
<td>-1.186</td>
</tr>
<tr>
<td></td>
<td>6.778</td>
</tr>
<tr>
<td>Tumor necrosis factor (ligand) superfamily, member 11</td>
<td>2.534</td>
</tr>
<tr>
<td></td>
<td>11.672</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 10a</td>
<td>-27.778</td>
</tr>
<tr>
<td></td>
<td>14.058</td>
</tr>
<tr>
<td><strong>Ag presentation</strong></td>
<td></td>
</tr>
<tr>
<td>Major histocompatibility complex, class II, DR beta 3</td>
<td>1.093</td>
</tr>
<tr>
<td></td>
<td>2.191</td>
</tr>
<tr>
<td>Major histocompatibility complex, class II, DR beta 4</td>
<td>3.791</td>
</tr>
<tr>
<td></td>
<td>4.271</td>
</tr>
<tr>
<td>Major histocompatibility complex, class II, DR beta 5</td>
<td>-1.258</td>
</tr>
<tr>
<td></td>
<td>6.135</td>
</tr>
</tbody>
</table>

**Table 2. Macrophage transcriptome in response to rBCG-hcs.** The values (averages of 2
independent experiments) represent fold changes in gene expression in THP-1 cells infected with BCG relative to control non-infected cells (BCG and control) and fold changes in gene expression of cells infected with rBCG-hcs relative to cells infected with BCG (rBCG-hcs and BCG). The red color represents increases and green decreases in gene expression, where white represents no significant changes.

3.3 Chromosomal expression of human active CatS in BCG

Due to the unstable nature of episomal vector backbones [172,173] and perhaps the choice of heterologous gene of interest [174], we have deemed the previous construct a potential liability especially for immunogenic studies in mice, which require stable expression of exogenous proteins in recombinant bacteria. Therefore, we opted for the integrative vector pJAK1.A to allow for chromosomal expression of CatS in BCG. Thus, pJAK1.A carrying a DNA segment corresponding to CatS active domain fused to the signal sequence of mycobacterial protein Ag85B (Fig. 7A) was electroporated into competent BCG organisms to obtain a novel recombinant strain referred to as “rBCG-CatS”. Selected transformant clones were grown and subjected to Western blot analyses of cell surface-associated proteins and results obtained showed conclusively the expression of substantial levels of CatS in rBCG-CatS (Fig. 7B). This novel recombinant strain was used to confirm key data obtained with rBCG-hcs and to develop the current study.
Figure 7. Construction and characterization of recombinant BCG expressing CatS. (A) Schematic of the CatS vector construct. Gene segment consisting of the mycobacterial Ag85B (fbpB) signal sequence and a portion of the mature Ag85B, together with the E. coli hemolysin A (hlyA) sequences and the human active Cathepsin S are synthesized. This segment is outfitted with attB1 and attB2, and was cloned using recombination technology into the pJAK1.A vector. (B) Characterization of rBCG-CatS. Western blot analysis of engineered rBCG-CatS strain demonstrating expression of CatS on the bacterial surface.

3.4 rBCG-CatS induces the expression of macrophage pro-apoptotic genes

Since apoptosis is an important cellular trait providing a link between innate and adaptive immunity in infected macrophages [55,175], we analyzed the expression level of 3 selected pro-apoptotic genes by reverse transcription quantitative PCR (RT-qPCR), BAD, DAP, and Puma; and 3 genes associated with apoptosis inhibition, AATF, Bcl-2, and Mcl-1. Consistent with the microarray findings, RT-qPCR data (Fig. 8) showed that expression level of anti-apoptotic genes is significantly higher in macrophages infected with BCG relative to those infected with rBCG-CatS. In particular, BCG-infected cells showed ~200-fold increase in the expression level of Mcl-1, when compared with cells infected with rBCG-CatS. In contrast, pro-apoptotic genes were highly expressed in cells infected with rBCG-CatS; most notably in DAP, where the fold increase was ~4-fold compared to BCG-infected cells. These
RT-qPCR data suggest that the novel rBCG-CatS would likely induce macrophage apoptosis and by doing so, improves antigen presentation.
Figure 8. Expression level of select apoptosis genes in RAW 264.7 cells infected with BCG or rBCG-CatS. Total RNA was isolated post overnight infection, and 1 μg of each
RNA sample was first reverse transcribed into cDNA, prior to RT-qPCR with gene-specific primers. ΔΔCT method was used for data analysis and the relative quantification was calculated using uninfected RAW 264.7 cells as point of reference. Statistical values were analyzed using one-way ANOVA with data obtained from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; ns = not significant.

3.5 Recombinant Cathepsin S protein induces active-caspase 3

Prior to examining the effect of rBCG-CatS on macrophage apoptosis, we first examined whether CatS activity is directly regulating it. Thus, macrophages were infected with latex beads coated with recombinant CatS protein or BSA for overnight then levels of active caspase-3 were determined by intracellular staining with specific antibodies and flow analyses. Results obtained showed that compared to cells exposed to BSA-coated beads, those ingested CatS-beads presented a marked increase in level of cellular apoptosis, as evidenced by the significant shift of fluorescent histograms corresponding to the amounts of cleaved caspase-3 (Fig. 9A), which reflects 28.8 ± 4.83% increase in apoptotic cells (Fig. 9B). These data are consistent with previous findings that IFNγ-induced apoptosis in lung epithelial cells is largely mediated by CatS [176].
Figure 9. CatS-coated beads induce active caspase-3 expression in RAW 264.7. Latex beads were coated with recombinant CatS protein or BSA for 3 h at RT, internalized by RAW 264.7 macrophages, and stained intracellularly with active caspase-3-specific antibody after an overnight incubation. Staurosporine (STP) is used to induce active caspase-3 expression in the positive control. Results are presented as (A) histogram and (B) bar graph of active caspase-3-positive events relative to untreated cells.

3.6 BCG prevents macrophage apoptosis while rBCG-CatS induces it

Prior to demonstrating that CatS delivery by BCG would reproduce data obtained with coated beads, we first sought to clarify the effect of BCG on macrophage apoptosis, as it was previously reported to be a poor inducer [177]. This time we performed Western blot analysis of cleaved caspase-3 levels and were unable to detect even a minor change in response to various BCG MOIs (Fig. 10). Taken one step further, we found that actually BCG actively prevents macrophage apoptosis response to powerful cell death inducers such as staurosporine (Fig. 11).
Figure 10. BCG does not induce active caspase-3 in the cell. RAW 264.7 macrophages were infected with BCG at different MOIs for overnight. Cells were then harvested and lysed; cell lysates were resolved on gel electrophoresis and probed with caspase-3 antibody. Staurosporine (STP) is used to induce active caspase-3 expression in the positive control.
Fig. 11. BCG inhibits caspase-3 activation in the presence of staurosporine in RAW 264.7 cells. Cells were infected with BCG at MOI 20 for overnight, then 25 nM staurosporine was added to the culture media for an additional overnight incubation. Cells were then lifted and stained for intracellular active caspase-3. Results are presented as (A) histogram and (B) bar graph of active caspase-3-positive events relative to untreated cells.

Thus one might suggest that our recombinant BCG is unlikely to activate the process of apoptosis since it would also block the effect of CatS that it delivers into macrophages. To exclude this possibility, BMDM were infected with rBCG-CatS or BCG and subjected to apoptosis analyses. Flow cytometry data showed ~1.5-fold increase in cleaved caspase-3 in cells infected with rBCG-CatS relative to those infected with BCG (Fig. 12A). Similarly, images from fluorescent microscopy examination of cell membrane integrity showed substantial levels of annexin V-positive cells in response to rBCG-CatS infection (Fig. 12B). In normal viable cells, phosphatidylserine (PS) is located on the cytoplasmic side of the cell membrane, retained there via an active process. When the cell undergoes apoptosis, the retention is lost and PS is exposed to the extracellular environment, allowing detection by annexin V. Taking together, these data indicate that if overnight exposure to BCG blocks staurosporine-induced apoptosis, the recombinant BCG is not able to oppose the effect of
instantaneous CatS delivery occurring during infection. Therefore, pro-apoptotic rBCG-CatS holds potential in reversing macrophage function defects caused by infection with conventional BCG.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>12.2</td>
</tr>
<tr>
<td>BCG</td>
<td>17.1</td>
</tr>
<tr>
<td>rBCG-CatS</td>
<td>23.3</td>
</tr>
</tbody>
</table>
Figure 12. rBCG-CatS induces cellular apoptosis. (A) Cells were infected at MOI 20 for 24 h prior to fixation/permeabilization and intracellular staining for active caspase-3. Flow analyses showed increased apoptosis in rBCG-CatS-infected BMDM relative to those infected with BCG. (B) RAW 264.7 cells were infected at MOI 20 with BCG strains expressing GFP for 24 h prior to staining with AF568-conjugated annexin V. Confocal microscopy images showed abundant annexin V-positive events (indicative of cells undergoing apoptosis) in rBCG-CatS-infected cells relative to those infected with BCG.

3.7 CatS expression in BCG improves its immunogenicity

The findings that BCG expressing CatS induces phagosome maturation and antigen presentation in macrophages [167], along with my demonstration of the pro-apoptotic feature, suggest that rBCG-CatS would be more immunogenic compared to the parental strain. To test this hypothesis, C57BL/6 mice were subcutaneously vaccinated with BCG strains and sacrificed 4 weeks later to analyse specific in vivo immune response to BCG antigens. Single splenocytes were prepared and subjected to staining with MHC II-restricted PE-conjugated I-A\(^b\)-P25 tetramers and flow analyses to determine the frequency of antigen 85B-specific CD4\(^+\)
T-cells. P25 is known to be a dominantly recognized epitope in mice during mycobacterial infection [178] and was shown to be recognized following BCG vaccination [179]. Results obtained (Fig. 13) shows a large expansion (~2-fold increase) of P25-specific CD4+ T-cells in rBCG-CatS vaccinated mice, compared to those injected with BCG. The degree of clonal expansion of antigen-specific CD4+ T-cells was also explored by a proliferation assay using ex vivo re-stimulation of CFSE-stained splenocytes with whole BCG lysate (WBL) (Fig. 14). Stimulated cells were allowed to proliferate for 96 h prior to flow cytometry quantification of CFSE signal decrease in gated CD4+ T-cells, indicative of cell divisions. Dot plots presented in Fig. 14A showed a near 2-fold expansion of re-stimulated CD4+ T-cells isolated from mice inoculated with rBCG-CatS, relative to those isolated from animal injected with PBS. Whereas a minor proliferation was observed in cells isolated from BCG vaccinated mice. In other experiments we determined the frequencies of cytokine-producing cells in response to ex vivo re-stimulation by means of intracellular staining for cytokines and flow analyses. Results obtained (Fig. 15) showed that 1.65% ± 0.40 CD4+ T-cells from rBCG-CatS-immunized animals produced IFNγ, compared to only 0.43% ± 0.24 in splenocytes isolated from mice vaccinated with BCG. Similarly, a higher number (1.39% ± 0.14%) of TNFα producing CD4+ T-cells was observed in cells isolated from rBCG-CatS-immunized animals compared to TNFα positive events (0.87% ± 0.046) in the BCG group (Fig. 16). As well, levels of IL-2 producing CD4+ T-cells (Fig. 17) was significantly higher (0.73% ± 0.15%) in rBCG-CatS group relative to the BCG group (0.37% ± 0.036). The role of CD8+ T-cells in TB immunity is not negligible [180-182] and therefore we also quantified cytokine-producing cells in this subset within WBL-re-stimulated splenocytes. Flow data showed that the frequencies of IFNγ-, TNFα-, and IL-2-producing CD8+ T-cells (1.45%, 0.81%, 0.27%,
respectively) are noticeably higher in cells isolated from rBCG-CatS-vaccinated mice than those from the BCG group (1.08%, 0.46%, 0.15%, respectively).

Figure 13. Ag85+ CD4+ T-cells generated in C57BL/6 mice immunized with rBCG-CatS. rBCG-CatS group showed improved tetramer staining of Ag85-specific CD4+ T-cells compared to BCG group, as shown in (A) 2-panel dot plots displaying the average frequencies of Ag85-positive cells ± SD in the CD4+ population. Data shown here are representative of 3 independent experiments. The data expressed in bar graph in (B) displays the average frequencies of Ag85-positive cells in the CD4+ population, with their statistical significance. **P<0.01; ns = not significant.
Figure 14. CD4+ T-cells proliferation upon ex vivo re-stimulation from C57BL/6 mice immunized with rBCG-CatS. rBCG-CatS group showed improved CD4+ T-cell proliferation compared to BCG group, as demonstrated by (A) 2-panel dot plots displaying the average frequencies of CFSE-signal ± SD in the CD4+ population. Data shown here are representative of 3 independent experiments. The data expressed in bar graph in (B) displays the average frequencies of CFSE-diluted cells in the CD4+ population, with their statistical significance. ***P<0.001; ns = not significant.
Figure 15. Intracellular cytokine expression of IFNγ upon ex vivo re-stimulation from C57BL/6 mice immunized with rBCG-CatS. rBCG-CatS group showed improved IFNγ production in CD4+ and CD8+ T-cell compared to BCG group, as demonstrated by (A) 2-panel dot plots displaying the average frequencies of cytokine-producing cells ± SD in the CD4+ population, and the average frequencies in the CD8+ population. The CD4+ data shown here are representative of 3 independent experiments, and the CD8+ data shown are representative of 2 independent experiments. The data expressed in bar graph in (B) displays the average frequencies of cytokine-producing cells in the CD4+ population, with their
statistical significance (left panel). **P<0.01; ns = not significant. The right panel displays the average frequencies of cytokine-producing cells in the CD8⁺ population from an average of 2 independent experiments.

Figure 16. Intracellular cytokine expression of TNFα upon ex vivo re-stimulation from
**C57BL/6 mice immunized with rBCG-CatS.** rBCG-CatS group showed improved TNFα production in CD4+ and CD8+ T-cell compared to BCG group, as demonstrated by (A) 2-panel dot plots displaying the average frequencies of cytokine-producing cells ± SD in the CD4+ population, and the average frequencies in the CD8+ population. The CD4+ data shown here are representative of 3 independent experiments, and the CD8+ data shown are representative of 2 independent experiments. The data expressed in bar graph in (B) displays the average frequencies of cytokine-producing cells in the CD4+ population, with their statistical significance (left panel), **P<0.01, ***P<0.001. The right panel displays the average frequencies of cytokine-producing cells in the CD8+ population from an average of 2 independent experiments.
Figure 17. Intracellular cytokine expression of IL-2 upon *ex vivo* re-stimulation from C57BL/6 mice immunized with rBCG-CatS. rBCG-CatS group showed improved IL-2 production in CD4+ and CD8+ T-cell compared to BCG group, as demonstrated by (A) 2-panel dot plots displaying the average frequencies of cytokine-producing cells ± SD in the CD4+ population, and the average frequencies in the CD8+ population. The CD4+ data shown here are representative of 3 independent experiments, and the CD8+ data shown are representative of 2 independent experiments. The data expressed in bar graph in (B) displays the average frequencies of cytokine-producing cells in the CD4+ population, with their statistical significance (left panel). *P<0.05; ns = not significant. The right panel displays the average frequencies of cytokine-producing cells in the CD8+ population from an average of 2 independent experiments.

Taken together, our data clearly demonstrate that CatS expression in BCG improves significantly its immunogenicity in terms of induction of specific cytokine secreting CD4+ and CD8+ T-cells.
CHAPTER 4: RECOMBINANT BCG EXPRESSING CASPASE-3 FURTHER IMPROVES VACCINE IMMUNOGENICITY

4.1 Background

The improved apoptosis and increased murine immunogenicity achieved by rBCG-CatS has inspired us to take on a more direct approach – to engineer a rBCG that expresses active caspase-3 (rBCG-C3). While CatS likely acts on players in the apoptotic pathway that ultimately lead to the activation of caspase-3, having a rBCG that expresses the executioner caspase might prove beneficial in the kinetics of immune response. Gartner et al. has engineered a tuberculosis DNA vaccine that expresses caspase-3 as part of the construct and demonstrated heightened immune response in the mice [183]. However, given the limited Mtb antigen sequences that a DNA construct could carry along with caspase-3, we decided to express caspase-3 in live BCG to allow presentation of a plethora of bacterial antigens to the lymphocytes, aiming to induce a more robust immunity.

4.2 Characterization of rBCG-C3

Given the success of the rBCG-CatS construct in terms of active expression and secretion, we have decided to utilize the same cloning strategy for rBCG expressing caspase-3. Since caspase-3 is normally synthesized as an inactive zymogen in the cell, in order to create a vector that expresses constitutively active caspase-3, we followed the footsteps of Srinivasula et al. [166] and constructed the gene in the “reverse caspase-3” manner. In short, the large and small subunit of caspase-3 sequence is reversed, and completed with a linker sequence in between [166]. This results in spontaneous folding of the molecule and produces an active protein [166]. Using the recombination-based vector, pJAK1.A, caspase-3 was
synthesized in place of Cathepsin S alongside with the signal sequence of mycobacterial protein Ag85B and the *E. coli* alpha-hemolysin secretion cassette to aid secretion [184] (Fig. 18A).

To validate expression and secretion of caspase-3 once transformed into BCG, cell wall proteins were dissociated and subjected to Western blotting for caspase-3. Fig. 18B shows the presence of caspase-3 in rBCG, whereas the absence is seen in BCG and BCG with vector control.

**Figure 18. Construction and characterization of recombinant BCG expressing active caspase-3.** (A) Schematic of the caspase-3 vector construct. Gene segment consisting of the mycobacterial Ag85B (*fbpB*) signal sequence and a portion of the mature Ag85B, together with the *E. coli* hemolysin A (*hlyA*) sequences and caspase-3 are synthesized. This segment is outfitted with *attB1* and *attB2*, and was cloned using recombination technology into the pJAK1.A vector. (B) Characterization of rBCG-C3. Western blot analysis of engineered
rBCG-C3 strain demonstrating presence of C3 on the bacterial surface. Recombinant BCG expressing vector control is denoted as E361. Hsp65 is probed as the loading control.

4.3 Human and murine cell lines infected with rBCG-C3 showed increased level of apoptosis

Since rBCG-C3 expresses caspase-3, examining the level of active caspase-3 in infected macrophages to gauge its level of apoptosis would be inconclusive. Thus, THP-1 cells were infected with rBCG-C3 and the level of apoptosis was evaluated by examining the degree of PARP cleavage. Full length PARP, or poly(ADP-ribose) polymerase, is a protein involved in DNA repair [185]. During apoptosis, PARP is cleaved by caspase-3 and thus rendered inactive and cell death proceeds [185]. Western blot analysis showed that while cell infected with BCG and BCG carrying vector control showed similar level of PARP cleavage to that observed in uninfected cells, a higher amount of cleaved PARP was detected in rBCG-C3-infected cells, suggesting that rBCG-C3 is pro-apoptotic (Fig. 19A). The pro-apoptotic potential of rBCG-C3 was further confirmed by epifluorescent microscopy of annexin V staining (Fig. 19B). Indeed, RAW cells infected with rBCG-C3 showed massive green fluorescence events, indicating heightened levels of phosphatidylserine surface expression. Taking together, these experiments demonstrated that rBCG-C3 is effective in inducing host cell apoptosis.
Figure 19. rBCG-C3 induces cellular apoptosis in vitro. (A) THP-1 cells were infected with rBCG-C3 at MOI 20 for 48 h. Cells were then harvested and cell lysates were prepared and subjected to SDS-PAGE and Western blotting with PARP antibody. (B) RAW 264.7 were infected with BCG strains expressing dsRed on an episomal vector (MOI 20), for 48 h...
prior to staining with AF488-conjugated annexin V and confocal microscopy analyses. Macrophages infected with rBCG-C3 show increased annexin V staining, indicating the levels of PS, on the cell surface.

4.4 Improved immunogenicity of rBCG-C3 infected mice

Similar to rBCG-CatS, rBCG-C3 was subjected to immunogenicity studies to elucidate the link between apoptosis and adaptive immunity. Female 4-6 weeks old C57BL/6 mice were injected subcutaneously at the neck scruff with 10^6 bacteria and were sacrificed at 3-4 weeks post-infection to examine the induced immune response in the spleen. Ag85B-specific MHC II-restricted tetramer staining showed that a larger expansion of CD4^+ specific T-cells (approximately 3.5-fold increase) occurred in mice vaccinated with rBCG-C3 relative to T-cell expansion in mice inoculated with BCG (Fig. 20A). Increase in adaptive immunity is undeniably confirmed by the level of T-cell proliferation after ex vivo re-stimulation with BCG WBL since CFSE-dilution experiments (Fig. 20B) showed substantial levels of CD4^+ and CD8^+ cell proliferation in rBCG-C3 test group (4.5- and 6.5- fold increase, respectively) when compared to the BCG test group.

Besides T-cell proliferation, functional read-outs were also examined by intracellular cytokine staining of ex vivo re-stimulated splenocytes. While tetramer staining only identifies a small set of T-cells with a single MHC-peptide specificity, intracellular cytokine staining reveals the sum total of T-cell response to the whole BCG bacterium. Compared to BCG test group, the results showed a substantial increase in the frequencies of IFN\(\gamma\) producing cells in both CD4^+ (9-fold increase) and CD8^+ (6-fold increase) subsets in rBCG-C3 samples (Fig. 20C). To a lesser degree, increases in the frequency of TNF\(\alpha\) producing CD4^+ and CD8^+ cells in rBCG-C3 samples were 1.5-fold and 2.3-fold, respectively, compared to BCG.
samples. Secreted IL-2 levels were also examined as a means to evaluate antigen-specific T-cell proliferation upon \textit{ex vivo} re-stimulation and the results (Fig. 20D) showed that rBCG-C3 splenocyte samples secreted 2.7-fold more IL-2 than the BCG sample.

In summary, improved immunogenicity \textit{in vivo} can be achieved using caspase-3 expressing BCG to enhance host cell apoptosis. This is illustrated by the increase in MHC-antigen specific CD4$^+$ T-cells, \textit{ex vivo} CD4$^+$ and CD8$^+$ T-cell expansion, and functional production of various cytokines.

\textbf{Figure 20. Immunogenicity studies in C57BL/6 mice.} (A) Tetramer staining showing Ag85-specific CD4$^+$ T-cell response. rBCG-C3 shows increased number of Ag85B-specific CD4$^+$ T-cells compared to BCG group. Values shown in the 2-panel dot plots are representative of 2 independent experiments (3 mice per experiment).
(B) CD4$^+$ T-cell proliferation upon *ex vivo* re-stimulation with BCG whole bacterial lysate. rBCG-C3 showed increased level of CD4$^+$ T-cell proliferation upon *ex vivo* re-stimulation, compared to BCG group. Values shown in the 2-panel dot plots are representative of 2 independent experiments (3 mice per experiment).
Intracellular cytokine expression shows functional CD4+ and CD8+ T-cells. Ex vivo re-stimulation with BCG whole bacterial lysate revealed that mice infected with rBCG-C3 has
heightened intracellular IFNγ and TNFα cytokine production. Values shown in the 2-panel dot plots are representative of 2 independent experiments (3 mice per experiment).

(D) Culture supernatant was retrieved from splenocytes stimulated with BCG whole bacterial lysate after an overnight incubation period and was subjected to IL-2 ELISA analysis. The assay shows that mice infected with rBCG-C3 has noticeably higher levels of IL-2 secretion compared to those infected with BCG. The data expressed displays the averages of triplicate wells, where the bar graphs are representative of 2 independent experiments (3 mice per experiment).

Since both rBCG-CatS and rBCG-C3 improves macrophage apoptosis and induce a strong immune response in vivo, one would like to know whether one strain is more potent than the other. Comparative immunogenicity studies were performed and the results obtained (Fig. 21A) showed that while rBCG-CatS still induces a higher (1.4-fold increase) Ag85B-specific CD4+ T-cell expansion in the mouse, relative to BCG, animal response was further improved (3-fold increase) in mice immunized with rBCG-C3.
A similar trend can be observed in cytokine production during *ex vivo* re-stimulation between the two recombinant BCG strains (Fig. 21B). rBCG-CatS was able to elicit more IFNγ and TNFα producing T-cells, compared to BCG. The only exception being the frequencies of TNFα-producing cells in the CD8+ subsets, which are identical. However, frequencies of cytokine-producing cells in the rBCG-C3 test group were consistently higher. Most notably, and relative to BCG, we observed a 7-fold increase in IFNγ producing CD8+ T-cells in the rBCG-C3 test group and only 1.6-fold increase in the rBCG-CatS group. Similarly, ELISA data (Fig. 21C) showed that, relative to BCG, rBCG-C3 induces 3-fold increase in levels of IL-2 secretion whereas nearly 1.5-fold increase was observed in response to rBCG-CatS.

**Figure 21.** Comparative immunogenicity studies of rBCG-CatS vs rBCG-C3. (A) Tetramer staining of Ag85-specific CD4+ T-cell response. rBCG-C3 shows increased number of Ag85B-specific CD4+ T-cells compared to rBCG-CatS group. 3 mice were used in each treatment group.
(B) Intracellular cytokine staining shows functional CD4$^+$ and CD8$^+$ T-cells. *Ex vivo* re-stimulation with BCG whole bacterial lysate revealed that mice infected with rBCG-C3 has heightened intracellular IFN$\gamma$ and TNF$\alpha$ cytokine production compared to the rBCG-CatS group. 3 mice were used in each treatment group.
(C) Culture supernatant was retrieved from splenocytes stimulated with BCG WBL after an overnight incubation period and subjected to IL-2 ELISA analysis. The assay shows that mice infected with rBCG-C3 has noticeably higher levels of IL-2 secretion compared to those infected with rBCG-CatS and with BCG. 3 mice were used in each treatment group.

Taken together, this comparative study revealed a superior animal immune response to rBCG-C3 relative to rBCG-CatS, albeit both recombinant strains are more immunogenic than BCG.
CHAPTER 5: DISCUSSION

5.1 Expression of CatS in BCG converts it into a more immunogenic vaccine

Since the Geneva consensus that the 100-year old BCG vaccine has a limited efficacy in preventing TB disease [186], many investigators proposed novel and attractive strategies on how this vaccine may be improved, or perhaps replaced entirely. With regards to live vaccines, research efforts focused on creating viral delivery systems for selected Mtb antigens, genetically improved BCG strains and attenuated Mtb strains [42]. Although live weakened Mtb strains were found to induce protective TB immunity in animal models [187], there were legitimate skepticisms about their safety, especially for immunocompromised individuals [188].

BCG has proved to be safe, with more than 3 billion doses administered and a very low incidence rate of serious side effects. Therefore, improving its efficacy while maintaining its level of safety is being considered as the best option to develop an effective TB vaccine by many investigators [170,186,189]. One of the most promising reshaped BCG strain developed so far is AERAS-422, which over-expresses Ag85B, Ag85A and VAPB47 (Rv3407) along with perfringolysin – a cytolysin secreted by Clostridium perfringens – as an endosome escape mechanism enabling translocation of mycobacterial antigens into the cytoplasm [190]. Unfortunately, this vaccine was dropped from clinical development because of serious adverse events associated with perfringolysin [191]. Thus, currently, only the previously developed VPM1002 vaccine is prevailing in clinical trials (phase IIa). VPM1002 (or rBCGΔureC::hly) was designed to increase the efficacy of BCG by enhancing the CD8+ response to TB. rBCGΔureC::hly derives from a Urease C-deficient mutant BCG (BCGΔureC) strain [71] engineered to secrete listeriolysin (Hly). The goal is to induce an acidic
environment within the host cell under which Hly (a major virulence factor of *Listeria monocytogenes*) is hyper active. Similar to perfringolysin, Hly forms pores in the phagosomal membrane allowing for enhanced MHC I antigen cross-presentations [71,192]. Although VPM1002 successfully passed safety criterion in phase I clinical trial [72], one cannot exclude the possibility of similar side effects observed with AERAS-422 while undergoing final confirmation of safety and efficacy. Therefore, there is an obvious need to develop additional elaborated BCG vaccines.

Many studies, including from our laboratory, demonstrated that BCG, although attenuated relative to the parental *M. bovis*, has retained the capabilities to block phagosome maturation and antigen presentation [50,53,164,193]. This would explain, at least in part, its low protective efficacy. In particular, we found that BCG is able to down-regulate MHC II-dependent antigen presentation via IL-10-dependent inhibition of CatS expression [164], and this phenotype is reversed when infecting macrophages with BCG expressing CatS [167]. These exciting findings opened up a great opportunity for TB vaccine development.

Prior to further investigation of the CatS approach for a vaccinal purpose, we first examined global macrophage transcriptome in response to our first version of BCG expressing CatS (rBCG-hcs). The transcriptome profiles obtained identified several genes that are differentially expressed by rBCG-hcs-infected macrophages compared to infection with BCG. These microarray data revealed for the first time important functional properties of CatS that far exceed a simple contribution to Ii chain degradation [194] and uncover a yet unsuspected transcription factor-like activity regulating macrophage gene expression. This is reminiscent of Goulet *et al.* [126] findings that cathepsin L, a closely related lysosomal
protease to CatS, is capable of trafficking to the nucleus of NIH3T3 cells to cleave latent transcription factors, releasing active factors to initiate nuclear signaling.

A striking finding that emerged from microarray analyses was the complete reversal in the expression of genes related to cell death in macrophages infected with rBCG-hcs. Building upon these findings we generated a BCG strain expressing CatS (rBCG-CatS) and attempted to further investigate apoptosis response to BCG. We found that BCG not only lacks the property to induce apoptosis, as previously reported [55], but also actively blocks apoptosis induction by powerful pharmacological agents such as staurosporine. In contrast, rBCG-CatS proved to be a great inducer of apoptosis, which is an important macrophage feature, associated with the ability to initiate an effective immune response [55,192,195].

The ability of cathepsins to induce cellular apoptosis has been reported earlier [157]. More specifically, Zheng et al. found that IFNγ-induced apoptosis in epithelial cells is mediated by CatS and suggested that CatS might be responsible for the pathogenesis of IFNγ-induced alveolar remodeling and emphysema [176]. While this assumption raises a safety concern about human vaccination with rBCG-CatS, no external signs of side effects were observed during our animal studies and detailed organ necropsy of rBCG-CatS-vaccinated animals did not reveal any macroscopic sign of toxicity. It should also be noted that CatS expression and activation is a predicted response to IFNγ-stimulated APC [171], and that IFNγ production is a gold standard marker for effective anti-TB immunity [196,197]. Thus our approach, i.e. secretion of active CatS by the vaccine, would only partially mimic a natural physiological response to IFNγ.
The major parameter used to assess the immunogenicity of novel TB vaccines is the *in vivo* expansion of specific T-cells in response to immune-dominant antigens carried by the vaccine. This test is currently being greatly facilitated with the availability of MHC tetramer reagents, which allow direct measurements of antigen-specific T-cell numbers in vaccinated animals. Remarkably, as per tetramer staining, mouse immunization with the pro-apoptotic rBCG-CatS induced a large expansion (~3-fold increase) of Ag85B-specific CD4+ T-cells in rBCG-CatS, compared to vaccination with BCG. Furthermore, *ex vivo* re-stimulation of spleen cells with BCG antigens showed that proliferating CD4+ T-cells from animals vaccinated with rBCG-CatS have the potential to secrete higher levels of a plethora of intracellular cytokines – IFNγ, TNFα, and IL-2, which is generally recognized as strong correlates with the protective potential of induced T-cells in vaccinated animals and humans [42].

While CD4+ T-cells are known to be essential for TB immunity, a number of investigators have investigated the role of CD8+ T-cells and found that CD4+ T-cells effectively control low-dose infections, which correspond to early stages of infection, but CD8+ T-cells possess a unique activity that makes them essential during high-bacterial burdens at late stages of infection [198]. Similar to CD4+, CD8+ T-cells have been shown to function mainly by producing IFNγ and by targeting infected cells for cytolysis [199,200]. However, BCG was shown to be insufficient in stimulating a robust CD8+ T-cell response [201] and more precise studies showed that mycobacterial antigen peptide recognition by MHC I-restricted T-cells is mediated by a TAP1-independent mechanism [202]. Interestingly, CatS has been shown to play an important role in generating peptides for a TAP1-independent pathway that is blocked in macrophage isolated from TAP−/−Cat S−/− mice, but reversed when
cells were complemented with recombinant CatS [203]. Therefore, as CatS is inhibited in macrophages infected with BCG, the TAP-independent antigen cross-presentation is probably attenuated in these cells as well. In this study, we took matters one step further, and quantified cytokine-producing cells in the CD8+ T-cell subset. We found that the frequencies of IFNγ-, TNFα-, and IL-2-producing CD8+ T-cells are noticeably higher in cells isolated from rBCG-CatS-vaccinated mice than those from the BCG group, thus providing additional evidence for the importance of CatS in reshaping conventional BCG.

5.2 Proposed mechanism of cathepsin S promoting apoptosis

While CatB was shown to cleave procaspase-1 and -11 directly, it was not involved in apoptosis [204]. Indeed, in terms of cleavage of procaspases involved in apoptosis, cathepsins cleave neither the executioner caspases (caspase-3 or -7), nor the initiator caspases (caspase-8 or -9) [156]. This suggests that cathepsins play a role in apoptosis signaling independent of direct cleavage of caspases. Indeed, the first molecule discovered in a cell free lysate study to be cleaved by cathepsins is Bid [156]. It was found that cathepsin-cleaved Bid was able to punctuate mitochondrial membrane and cause cytochrome c release and subsequent caspase activation [156]. This finding was confirmed in various cell lines, including MCF-7, HaCaT, SH-SY5Y, Caco-2, and HepG2 cells [158]. In vitro experiments show that cathepsins (including CatS) cleaves and thereby inactivates anti-apoptotic molecules Bcl-2, Bcl-xL, and Mcl-1 [158]. The major inhibitor of caspases, XIAP, which regulates apoptosis downstream of mitochondria, is also a major cathepsin target, found in cell lines SH-SY5Y and CaCo-2 [158]. Evidence of potential involvement of CatS in apoptosis was provided by cell-free system studies showing that CatS cleaves recombinant Bcl-2, Bak, Mcl-1 and XIAP [158].
When Zheng et al. showed that CatS is involved in epithelial cell apoptosis, and is a critical event in the pathogenesis of IFNγ-induced alveolar remodeling and emphysema [176], they found that CatS induces increased mRNA level in extrinsic and intrinsic apoptosis genes (i.e. Fas, FasL, TNFα, TRAIL, Bak, Bid, Bim, caspase-3, -6, -8, -9, and PKCδ). Thus, CatS may be involved in apoptosis at the transcriptomic level. For example, in Fig. 8 we showed that cells infected with BCG expressed more apoptosis antagonizing transcription factor (AATF), while those infected with rBCG-CatS have diminished levels. AATF is induced by PERK-eIF2α signaling in response to ER stress, and then AATF transcriptionally activates the Akt1 gene through STAT-3, which sustains Akt1 activation and promotes cell survival [205]. CatS appears to be actively acting on the expression of AATF at the transcription level, to promote apoptosis in the cell.

5.3 Benefits of a recombinant BCG strain expressing executioner caspase

Currently, a caspase-3 vaccine against tuberculosis has been described in Gartner et al., in the form of a DNA vaccine [183]. In attempt to increase cross-presentation through apoptosis, this vaccine co-expressed secreted Ag85A and catalytically active caspase-3. This pro-apoptotic vaccine was shown to induce rapid apoptosis in HEK293T cells. Furthermore, in the murine model, vaccination triggered more Ag85A-specific IFNγ producing splenocytes, and more efficient IL-2 and IFNγ-producing memory cells in spleen and lungs after Mtb challenge. The addition of caspase-3 in a DNA vector, compared to vector carrying Ag85A alone, lowered lung bacteria burden, increased median survival time, thus increased protection after Mtb infection.
Compared to Gartner’s work, our rBCG-C3 also induced apoptosis, produced more Ag-specific T-cell response in the spleen, and more cytokine-producing T-cells upon re-stimulation. This is in concordance of the concept that a pro-apoptosis vaccine enhances antigen availability for processing and presentation by APCs. However, as we are using live attenuated vaccine, we would be able to generate a broader antigenic response, whereas the DNA vaccine is restricted to Ag85A-specific antigen presentations. Our work demonstrated a rBCG expressing C3 is able to generate a response to a wide range of BCG antigens, as shown by significant T-cell proliferation during *ex vivo* re-stimulation with total BCG antigens.

However, while comparing the function of rBCG-CatS and –C3, it was unexpected that rBCG-C3 would induce a stronger immune response than rBCG-CatS. This expectation is due to the fact that, in addition to induction of apoptosis, CatS also promotes MHC II maturation. One possibility to explain these discrepancies is that unlike caspase-3, CatS is not an executioner in the apoptosis pathway that promises the definitive fate of undergoing cell death.

### 5.4 Stability of endogenously expressed CatS and C3 in BCG

While it is promising that rBCG-C3 showed more effect in murine immunogenicity model compared to rBCG-CatS, we are still facing the hurdle of plasmid instability in BCG strains expressing foreign genes. Indeed, others have also observed the variable instability of *hsp60* promoter plasmids [173,174,206]. The stability of heterologous gene expression in BCG seems to be at least in part, dependent on the genetic compatibility between the expression cassette within the plasmid and the mycobacteria [206]. The *P*<sub>AN</sub> sequence, which
is a weak promoter derived from *M. paratuberculosis* [207], has been used for numerous foreign protein expression – namely, the β-galactosidase of *E. coli* [208], the Gp63 surface antigen of *Leishmania* [209], and the Nef antigen of Simian Immunodeficiency Virus [210]. Indeed, it is known that expressing heterologous proteins in rBCG poses a metabolic stress and can result in mutations in the hsp60 promoter, due to recombination between host and plasmid [211]. In other words, not every antigen is necessarily expressed or tolerated by BCG.

Therefore, we propose to solve the gene stability issue by a non-genetic manipulation method. In this context, I contributed significantly to a recent study in our laboratory by Liao et al. [168] that showed a promising method of decorating the surface of BCG with immunogenic proteins (Fig. 22). It is based on expression of proteins of interest in fusion with a mutant version of monomeric avidin protein using Gateway cloning described earlier. The resulting chimeric protein is able to bind reversibly to biotin. Upon BCG surface biotinylation, we can rapidly decorate the bacterial surface with avidin fusion protein. This method was proven to be stable and reproducible when tested with a surrogate ovalbumin antigen and mycobacterial protein ESAT6. Modification to the bacterial surface does not affect its growth in culture media, the survival within host cell, nor interfere with antigen presentation and loading onto MHC molecules. Furthermore, it was found that surface decorated BCG induced similar immune response as those genetically expressing the same antigen. This method opens the possibility of generating a rBCG strain that present CatS and C3 proteins, individually or in combination, which might further enhance the efficacy of BCG. To further improve the vaccine, one could also present one or multiple immunodominant mycobacterial proteins on a plasmid, which should be stable given its
homogeneous origin. This combination could be synergistic in effect, as the CatS molecule aids mature MHC II surface expression and apoptosis, while the apoptotic property is further optimized by the addition of C3. Any other addition of Mtb immuno-dominant antigen that is absent in BCG would be highly beneficial to development of vaccine immunity.

**Figure 22. Schematic of BCG surface decoration approach.** BCG surface is first biotinylated with hydrosoluble biotin, then exposed to purified avidin-fusion proteins. Due to the natural affinity of biotin-avidin, BCG can now be surface decorated with the antigen of interest. Adapted from Liao TY et al. PLoS ONE. 2015. 10(12):e0145833. Reprinted with permission from PLoS.
CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusion

The work in this thesis describes recombinant BCG strains that improved immunological response in the murine model via their pro-apoptotic ability. Canonically described as the protease that participates in MHC II maturation process, CatS was first discovered in our laboratory to be inhibited via a IL-10-dependent mechanism in the presence of BCG. Besides its role in antigen processing, it was elucidated from a microarray study that it participates in other cellular functions such as antigen presentation, phagosome maturation and apoptosis. Thus, we showed that cells infected with rBCG-CatS produced increased levels of apoptosis and induced a higher immune response in the mouse. With apoptosis in mind, we engineered a more direct approach – another strain of pro-apoptotic rBCG strain that expresses caspase-3, which reproduced the properties of rBCG-CatS but was shown to be more immunogenic. Based on work thus far, this project has the potential to diverge into pro-apoptotic BCG strains as promising TB vaccine candidates.

6.2 Future directions

To further examine the BCG strains in vaccine development, the safety of these bacteria must be evaluated using the BALB/c SCID mice model, which are mice characterized by an inability to mount an adaptive immune response. With the rise of co-infection of TB and HIV, it is imperative for vaccine candidates to be safe and effective in both healthy and immunocompromised individuals. Once the candidates have been deemed safe, protective efficacy of these vaccine strains can be evaluated using experimental TB models such as the mouse and guinea pig.
Since the main reason for BCG’s failure in being a successful vaccine is its waning ability of sustaining immunological memory [42], future work should focus on deciphering whether rBCG-CatS or –C3 promotes effector (T_{em}) or central memory (T_{cm}). While T_{em} produces effector cytokines such as IFN\(\gamma\), TNF\(\alpha\), and IL-2, the production of IL-2 declines in their final stage of differentiation and fail to continue in proliferation. In contrast, T_{cm} expresses high levels of CD62L and CCR7, but most importantly, abundant levels of IL-2 [212,213]. It is the IL-2\(^+\) T_{cm} population that is instrumental in long-term containment of Mtb infection [214]. Therefore, it is paramount that in future experimental TB studies, one must examine the longevity of memory elicited by this recombinant BCG strain.

In order to overcome the plasmid instability problem, as well as for the ability to introduce both CatS and C3 into the bacterium, it would be beneficial to exploit the avidin-decorating system, which would deliver both molecules to antigen presenting cells at the same time.

Taken together, the work presented in this thesis showed that pro-apoptotic BCG strains hold promise to a more effective tuberculosis vaccine. Further work includes examining the safety and protective efficacy of these strains, and to toggle the relative proportion of CatS and C3 presented by the vaccine.
REFERENCES


[19] [No authors listed]. (2000). Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999. *Am. J. Respir. Crit. Care Med.* 161, 1376-95.


Mycobacterium bovis BCG urease attenuates major histocompatibility complex class II 

W.H. (2004). Inhibition of major histocompatibility complex II expression and antigen 
processing in murine alveolar macrophages by Mycobacterium bovis BCG and the 19-

to T lymphocytes through MHC-I and CD1 in tuberculosis. Nat. Med. 9, 1039-46.

lipoarabinomannan antagonizes Mycobacterium tuberculosis-induced macrophage apoptosis 


reactions - Bacille Calmette-Guérin (BCG) vaccine.

administered BCG strains including an evolutionarily early strain and evolutionarily late 
strains of disparate genealogy induce comparable protective immunity against tuberculosis. 
Vaccine 27, 441-5.

Strains in Mice and Correlation With Genome Polymorphisms. Mol. Ther. 24, 398-405.

Vaccine Have Very Different Effects on Tuberculosis and on Unrelated Infections. Clin. 
Infect. Dis. 61, 960-2.

22, 249-57.


Science 281, 1305-8.

[95] Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., 
and Peter, M.E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form 
a death-inducing signaling complex (DISC) with the receptor. EMBO J. 14, 5579-88.


independent mitochondrial damage, a required first step for apoptosis. Immunity 22, 355-70.

[98] Elnemr, A., Ohta, T., Yachie, A., Kayahara, M., Kitagawa, H., Fujimura, T., Ninomiya, 
cells disable function of Fas receptors at several levels in Fas signal transduction pathway. 
Int. J. Oncol. 18, 311-6.

[99] Cheng, J., Zhou, T., Liu, C., Shapiro, J.P., Brauer, M.J., Kiefer, M.C., Barr, P.J., and 
Mountz, J.D. (1994). Protection from Fas-mediated apoptosis by a soluble form of the Fas 


tuberculosis strains evade apoptosis of infected alveolar macrophages. J. Immunol. 164, 
2016-20.


[103] Velmurugan, K., Chen, B., Miller, J.L., Azogue, S., Gurses, S., Hsu, T., Glickman, M., 

Apoptosis genes in human alveolar macrophages infected with virulent or attenuated 
Biol. 29, 545-51.

Pathogenic Mycobacterium tuberculosis evades apoptosis of host macrophages by release of 


97


molecules through IL-10-dependent inhibition of cathepsin S. *J. Immunol.* 175, 5324-32.


Mol. Microbiol. 6, 3331-42.


