



**Brunel**  
University  
London

**Investigating the role of conglutinin in host-  
pathogen interactions in bovine tuberculosis**

A thesis submitted in partial fulfilment of the requirements for the degree  
of Doctor of Philosophy

By

**Arshad Mehmood**

**Division of Biosciences**

**Department of Life Sciences**

**College of Health and Life Sciences**

**Brunel University London**

**February 2019**

## **Declaration**

I hereby declare that the research presented in this thesis is my own work, except where otherwise specified and has not been submitted for any other degree.

Arshad Mehmood

## Abstract

Bovine tuberculosis is an infectious disease mainly in livestock caused by *Mycobacterium bovis* and has had substantial economic impacts in the UK and worldwide. Innate immunity against bovine tuberculosis is not fully understood, and some of the first host molecules to be involved in innate immunity are collectins, which are soluble C-type lectins that play an important role in the targeting and clearance of microbes. Conglutinin is an important collectin, which is synthesized in the liver and is present in the serum and can be produced locally by neutrophil, dendritic and macrophage cells. Conglutinin is a unique collectin found in cattle and other grazing animals. Its biological role is not fully understood. It is therefore interesting to study conglutinin's role in the bovine infection and immunity. In this study, the aim was to investigate the role of conglutinin in bovine tuberculosis, by examining its influence in host-pathogen interactions between mycobacteria and macrophages.

A recombinant fragment of conglutinin (rfBC) composed of  $\alpha$ -helical neck region and the C-terminal CRD region was successfully expressed in *E. coli*, purified and characterised. It was observed that rfBC binds to mycobacteria (*M. bovis* BCG and *M. smegmatis*) in the presence of  $\text{Ca}^{2+}$  in a dose-dependent manner. A direct bacteriostatic effect for conglutinin was also observed inhibiting mycobacterial growth *in vitro*. This is the first time a bacteriostatic effect for conglutinin has been observed against Gram-positive bacteria.

It was also found that rfBC bound on the surface of mycobacteria (*M. bovis* BCG and *M. smegmatis*) also inhibited their phagocytosis by THP-1 macrophages cells. In this study we also observed that conglutinin led to dampening of cytokines and chemokines response in the THP-1 cells infected by *M. bovis* BCG and complement coated *M. bovis* BCG as compared to untreated *M. bovis* BCG. *In vivo*, macrophages phagocytose mycobacteria after entry in to the host but ultimately fail to destroy them and provide hostile environment for multiplication. Conglutinin inhibition of phagocytosis of these bacteria in to macrophages and dampening of pro-inflammatory response may be protective by keeping them extracellular where these can be easily eliminated by the host immune response.

## Table of Contents

<b>Declaration</b> .....	ii
<b>Abstract</b> .....	iii
<b>List of Figures</b> .....	x
<b>List of Tables</b> .....	xiii
<b>Acknowledgments</b> .....	xiv
<b>Chapter 1 : Introduction</b> .....	1
1.1 Bovine tuberculosis .....	2
1.2 Epidemiology of bovine tuberculosis.....	2
1.3 Incidence of bovine tuberculosis (BTB) in the United Kingdom .....	4
1.4 The role of badgers in the epidemiology of bovine tuberculosis .....	6
1.5 Characteristics of <i>M. bovis</i> .....	8
1.6 Transmission of <i>M. bovis</i> .....	9
1.7 Pathogenesis of bovine tuberculosis .....	9
1.7.1 Granuloma structure and function .....	11
1.8 Zoonotic impact of bovine Tuberculosis.....	13
1.9 Symptoms of bovine tuberculosis .....	13
1.10 Diagnosis of bovine tuberculosis .....	14
1.10.1 Tuberculin skin test (TST).....	14
1.10.2 Interferon gamma (INF- $\gamma$ ) release assay (IGRA) test .....	15
1.10.3 Post mortem diagnosis of <i>Mycobacterium bovis</i> infection.....	15
1.10.4 Histopathologic examination.....	15
1.11 Treatment and prevention of BTB .....	15
1.12 Biomarkers for Bovine tuberculosis.....	17

1.13 Innate immunity .....	19
1.14 Immune response against <i>M. bovis</i> .....	20
1.15 Role of complement system in bovine tuberculosis.....	21
1.16 Collectins and their roles in innate immunity .....	24
1.16.1 Receptor for collectins on immune cells .....	25
1.16.2 Surfactant protein A and D.....	26
1.16.3 Conglutinin .....	28
<b>Chapter 2: Materials and Methods</b> .....	<b>33</b>
2.1 Bacterial cell culture media.....	34
2.1.1 Luria broth (LB) media.....	34
2.1.2 Mycobacteria growth.....	34
2.2 Frozen storage of mycobacteria .....	34
2.3 Acid-Fast staining .....	35
2.4 Colony Forming Units (CFU) counts of mycobacteria.....	35
2.5 Thawing and culturing of THP-1 cells.....	36
2.6 Transformation of competent cells.....	36
2.6.1 Preparation of competent cells .....	36
2.6.2 Transformation of competent cells .....	37
2.6.3 Isolation of plasmid DNA.....	37
2.7 Protein expression .....	38
2.7.1 Pilot expression of recombinant conglutinin (BK-neck-CRD) and MBP (Maltose binding protein).....	38
2.7.2 Expression and purification of the recombinant conglutinin (rfBC) at large scale.....	39
2.7.3 Large scale expression and purification of maltose-binding protein (MBP) .....	40
2.7.4 Removal of endotoxin from rfBC.....	41

2.8 Protein characterization.....	41
2.8.1 SDS-PAGE .....	41
2.8.2 Western blot.....	42
2.8.3 Enzyme-linked immunosorbent assay (ELISA).....	43
2.8.4 Cross linking.....	44
2.9 Preparation of <i>M.smegmatis</i> and <i>M. bovis</i> BCG bacteria for Enzyme-linked immunosorbent assay (ELISA) for binding assays.....	44
2.9.1 rfBc binding with <i>M. bovis</i> BCG and <i>M. smegmatis</i> cells by ELISA.....	45
2.10 rfBC binding with <i>M. bovis</i> BCG cells microscopy.....	45
2.11 Direct effect of rfBC to <i>M. smegmatis</i> and <i>M. bovis</i> BCG growth.....	46
2.12 Measuring <i>M. bovis</i> BCG cell lysis by propidium iodide uptake .....	46
2.13 Infection Assays .....	47
2.13.1 Infection Assay by Colony forming Units (CFU).....	47
2.13.2 Infection assay by microscopy .....	49
2.14 Nuclear Factor Kappa B (NF-κB) Translocation Assay .....	50
2.15 Complement activation assay.....	51
2.15.1 Serum adsorption.....	51
2.16 Haemolytic assay.....	52
2.16.1 Preparation of neuraminidase-treated sheep erythrocytes (NE).....	52
2.16.2 Alternative pathway complement consumption assay.....	52
2.17 Complement dependent interactions of <i>M bovis</i> BCG coated with rfBC with THP-1 cells and phagocytosis by CFU (Colony forming Units). .....	53
2.18 Complement dependent interactions of <i>M. bovis</i> BCG coated with rfBC with THP-1 cells and phagocytosis by microscope.....	54
2.19 Quantitative real-time polymerase chain reaction (q-PCR) to determine gene expression.....	55
2.19.1 rfBC treatment of THP-1 cells with or without intracellular <i>M. bovis</i> BCG .....	55

2.19.2 Designing Primers for target genes .....	56
2.19.3 RNA extraction from THP-1 cells.....	56
2.19.4 DNase treatment .....	57
2.19.5 Synthesis of cDNA from total RNA.....	57
2.19.6 Quantitative real time PCR (qPCR).....	57
2.19.7 Data analysis.....	58
2.19.8 Multiplex analysis.....	58
<b>Chapter 3: Purification and characterization of recombinant fragment of bovine conglutinin (rfBC) expressed in <i>Escherichia coli</i> and its impact on the growth of <i>M. smegmatis</i>.....</b>	<b>60</b>
3.1 Introduction.....	61
3.2 Results.....	62
3.2.1 Expression and purification rfBC in <i>E. coli</i> (BL21 (DE3) pLysS strain) .....	62
3.2.2 Large scale expression of rfBC .....	63
3.2.3 Purification of rfBC.....	64
3.2.4 Western blot and ELISA analysis of rfBC for confirmation of protein by anti- conglutinin.....	65
3.2.4.1 Western blot analysis.....	66
3.2.4.2 ELISA to confirm the rfBC presence in purified fractions .....	68
3.2.5 Endotoxin level measurement (LAL assay) of rfBC .....	69
3.2.6 Cross-linking of rfBC.....	70
3.2.7 Expression and purification of maltose binding protein (MBP) in <i>E. coli</i> (BL21 (DE3) pLysS strain).....	71
3.2.8 Binding of rfBC to <i>M. smegmatis</i> in the presence of calcium and EDTA.....	74
3.2.9 Direct effect of rfBC on <i>M. smegmatis</i> growth.....	75
3.2.10 Effect of rfBC on uptake of <i>M. smegmatis</i> by THP-1 cells .....	76
3.3 Discussion.....	77

<b>Chapter 4- The role of conglutinin (rfBC) in mycobacterial infection. Insights from interactions with <i>M. bovis</i> BCG</b> .....	81
4.1 Introduction.....	82
4.2. Results.....	83
4.2.1: Binding of rfBC to <i>M. bovis</i> BCG .....	83
4.2.2: Binding of rfBC with <i>M. bovis</i> BCG by immunofluorescence microscopy .....	84
4.2.3 Direct effect of rfBC on <i>M. bovis</i> BCG growth.....	86
4.2.4 Fluorescence microscopy to study the direct effect of rfBC on the <i>M. bovis</i> BCG.....	87
4.2.5 <i>M. bovis</i> BCG cells stained by Propidium Iodide (PI) after direct effect of rfBC.....	88
4.2.6 Effect of rfBC on uptake of <i>M. bovis</i> BCG by THP-1 cells.....	89
4.2.7 Effect of rfBC on uptake of <i>M. bovis</i> BCG by THP-1 cell by fluorescence microscopy .....	91
4.2.8 The pro-inflammatory response of THP-1 macrophages during phagocytosis of <i>M. bovis</i> BCG treated with and without rfBC.....	93
4.2.10 Multiplex Array Analysis to study the cytokines, chemokines and growth factors production by THP-1 cells after phagocytosis <i>M. bovis</i> BCG treated with and without rfBC.....	96
4.2.10.1 Multiplex Array Analysis to study the cytokines production by THP-1 cells after phagocytosis <i>M. bovis</i> BCG treated with and without rfBC. ....	97
4.2.10.2 Multiplex Array Analysis to study the chemokines production by THP-1 cells after phagocytosis <i>M. bovis</i> BCG treated with and without rfBC.....	99
4.2.10.3 Multiplex Array Analysis to study the growth factors and related ligands and receptors production by THP-1 cells after phagocytosis <i>M. bovis</i> BCG treated with and without rfBC. ....	100
4.2.11 NF- $\kappa$ B cytoplasm to nucleus translocation during phagocytosis of <i>M. bovis</i> BCG (with and without rfBC) by THP-1 cells. ....	102
4.3 Discussion.....	104
<b>Chapter 5 – Investigating the effect of complement on the interactions of rfBC and <i>M. bovis</i> BCG and its implications for mycobacterial infection.</b> .....	111



5.1 Introduction.....	112
5.2 Results.....	113
5.2.1 Complement consumption assay with <i>M. bovis</i> BCG.....	113
5.2.2 Effect of rfBC on the phagocytosis of complement deposited <i>M. bovis</i> BCG by THP-1 cells.....	114
5.2.3 The effect of rfBC on the uptake of complement deposited <i>M. bovis</i> BCG by THP-1 cells after 3 hours by fluorescence microscopy.....	115
5.2.4 The pro-inflammatory response of THP-1 macrophages during phagocytosis of complement deposited <i>M. bovis</i> BCG treated with and without rfBC. ....	116
5.2.6 Multiplex array analysis to study cytokine, chemokine and growth factor production from THP-1 cells infected by <i>M. bovis</i> BCG treated with rfBC and complement and rfBC. ....	118
5.2.6.1 Production of pro-inflammatory and anti-inflammatory cytokines by THP-1 cells after 24 and 48 hours of the infection.....	118
5.2.6.2 Production of chemokines by THP-1 cells after 24 and 48 hours of the infection. ....	120
5.2.6.3 Production of growth factors and related ligands and receptors production by THP-1 cells after 24 and 48 hours of the infection.....	121
5.2.7 Production of rfBC treated complement deposited <i>M. bovis</i> BCG on NF- $\kappa$ B cytoplasm to nucleus translocation. ....	123
5.3 Discussion.....	124
<b>Chapter 6: Concluding Discussion and Future perspectives .....</b>	<b>131</b>
6.1 Conclusions and future perspectives.....	132
<b>Chapter 7: References .....</b>	<b>139</b>

## List of Figures

### Chapter 1

Figure. 1.1: Worldwide incidence of <i>M. bovis</i> BCG infection.....	4
Figure.1.2: Comparison of new incidences of <i>M. bovis</i> infection from 2018.....	6
Figure. 1.3: The life cycle of <i>Mycobacterium</i> and granuloma formation.....	12
Figure. 1.4: Innate and adaptive immunity interaction:.....	18
Figure 1.5: Schematic of complement pathways. ....	22
Fig. 1.6: Structure of the collectins. ....	24
Figure.1.7: Structure of bovine conglutinin: Conglutinin is an oligomer of polypeptide. ....	28

### Chapter 2

Figure 2.1: Preparation of 10-fold serial dilutions of bacteria.....	34
--	----

### Chapter 3

Figure 3.1: Pilot expression of recombinant bovine conglutinin rfBC in <i>E. coli</i> BL21 (DE3) pLysS. ....	60
Figure 3.2: Large-scale expression of rfBC. ....	61
Figure 3.3: rfBC purified fractions. ....	62
Figure 3.4 A: SDS-PAGE gel of rfBC and MBP for western blot analysis. ....	64
Figure 3.4 B: Western blot analysis of rfBC for confirmation of proteins by anti-conglutinin.....	65
Figure 3.5: ELISA data confirmed presence of rfBC in the purified fractions.....	66
Figure 3.6: LAL assay to measure endotoxin levels in the purified rfBC.....	67
Figure 3.7: Chemical cross-linking of purified rfBC to show that it could form dimeric and trimeric structures. ....	68
Figure 3.8A: Characterisation of MBP produced during pilot expression. ....	69
Figure 3.8 B: Characterisation of MBP produced during large-scale expression.....	70

Figure 3.8 C: Purified fraction of maltose binding protein (MBP). .....	71
Figure 3.9: Binding of rfBC to <i>M. smegmatis</i> . .....	72
Figure 3.10 : Direct Effect of rfBC on <i>M. smegmatis</i> growth. ....	73
Figure 3.11: Effect of rfBC on the uptake of <i>M. smegmatis</i> by THP-1 cells. ....	74
<b>Chapter 4</b>	
Figure 4.1: Serial dilution of rfBC with <i>M. bovis</i> BCG in the presence of 5mM Ca <sup>2+</sup> and 5mM EDTA buffers .....	82
Figure 4.2: Binding of rfBC with <i>M. bovis</i> BCG by immunofluorescence microscopy.....	84
Figure 4.3 : Direct Effect of rfBC to <i>M. bovis</i> BCG . ....	85
Figure 4.4: Fluorescence microscopy of <i>M. bovis</i> BCG stained with propidium iodide. ....	86
Figure 4.5: Propidium iodide (PI) assay to assess bacterial cell lysis as a result of incubation with rfBC. ....	87
Figure 4.6: Effect of recombinant rfBC on the uptake of <i>M. bovis</i> BCG by THP-1 cells. ....	89
Figure 4.7: Microscopic representation of effect of rfBC on the phagocytosis of <i>M. bovis</i> BCG by THP-1 cells. ....	91
Figure 4.8: Expression of pro-inflammatory cytokines by THP-1 cells treated with <i>M. bovis</i> BCG and different concentrations of rfBC. ....	93
Figure 4.9: Expression of anti-inflammatory cytokines by THP-1 cells treated with <i>M. bovis</i> BCG and different concentrations of.....	94
Figure 4.10: Multiplex cytokine analysis of supernatants collected at 24 h after phagocytosis of <i>M. bovis</i> BCG by THP-1 macrophages treated with rfBC.....	97
Figure 4.11: Multiplex chemokines analysis of supernatants collected at 24 h after phagocytosis of <i>M. bovis</i> BCG by THP-1 macrophages treated with rfBC. ....	98
Figure 4.12: Multiplex analysis of growth factors and related ligands and receptors supernatants collected at 24 and 48h after phagocytosis of <i>M. bovis</i> BCG by THP-1 macrophages treated with rfBC. ....	100

Figure 4.13: NF- $\kappa$ B translocation in THP-1 cells after uptake of rfBC bound <i>M. bovis</i> BCG. ....	102
---	-----

## Chapter 5

Figure 5.1: Activation of alternative pathway by <i>M. bovis</i> BCG in normal human serum.....	113
---	-----

Figure 5.2: Effect of rfBC on the phagocytosis of complement coated <i>M. bovis</i> BCG by THP-1 cells.....	114
---	-----

Figure 5.3: Fluorescence microscopy showing the effect of rfBC on the phagocytosis of complement deposited <i>M. bovis</i> BCG by THP-1 cells.....	115
--	-----

Figure 5.4: Expression of pro-inflammatory cytokines by THP-1 cells treated with complement deposited <i>M. bovis</i> BCG and different concentrations of rfBC. ....	116
--	-----

Figure 5.5: Expression of anti-inflammatory cytokines by THP-1 cells treated with complement deposited <i>M. bovis</i> BCG and different concentrations of rfBC: .....	117
--	-----

Figure 5.6: Multiplex cytokine analysis of supernatants collected at 24 h after phagocytosis of complement deposited <i>M. bovis</i> BCG by THP-1 macrophages treated with/without rfBC. ....	119
---	-----

Figure 5.7: Multiplex chemokines analysis of supernatants collected at 24 h after phagocytosis of complement deposited <i>M. bovis</i> BCG by THP-1 treated with rfBC.....	120
--	-----

Figure 5.8 : Multiplex analysis of growth factors and related ligands and receptors supernatants collected at 24 and 48h after phagocytosis of complement deposited <i>M. bovis</i> BCG by THP-1 macrophages treated with rfBC. ....	122
--	-----

Figure 5.9: NF- $\kappa$ B translocation in THP-1 cells after uptake of <i>M. bovis</i> BCG treated with rfBC and complement plus rfBC. ....	123
--	-----

## List of Tables

### Chapter 2

Table 2.1: Composition of SDS-PAGE loading sample buffer (2X Treatment buffer) .....	36
Table 2.2: Components of resolving gel for SDS-PAGE.....	39
Table 2.3: Components of stacking gel for SDS-PAGE.....	40
Table 2.4: DNA Sequence for forward and reverse primers used for amplification of different target genes in THP-1 cells. All sequences are 5' to 3'.....	54

## **Acknowledgments**

A huge thank you to my principle supervisor Dr Anthony Tsolaki for his support, encouragment and guidance throughout my PhD. I could not have asked for a better supervisor.

I am greatly indebted to my second supervisor, Dr Uday Kishore for his guidance and kind advice through out this project.

I would like to thanks Dr Ansar Pathan for his help in this project.

My thanks to all my labortory colleagues, especially Muhammad Suleman Riaz, Anuvinder Kaur and Basu Dev Paudyal for their help and for the good time to spend with them in a labortory.

A special thanks to my wife for her invaluable contributions through out this period.

I would also like to say a heartfelt thanks to my parents for their unconditional support, especially my mother who always have believe in me and gave me a lot of encouragment during this period. I could not have done any of this without their prayers.

## List of Abbreviations

ADC	Albumin Dextrose Catalase
APC	Antigen Presenting Cell
BCG	Bacillus Calmette Guerin
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BS3	bis(sulfosuccinimidyl)suberate
BTB	Bovine tuberculosis
CaCl <sub>2</sub>	Calcium Chloride
cDNA	Complementary DNA
CFP 10	Culture Filtrate Protein 10
CFU	Colony Forming Unit
CO <sub>2</sub>	Carbon dioxide
CR	Complement receptor
CRD	Carbohydrate recognition domain
cRPMI	Complete Roswell Park Memorial Institute medium
DAB	3'3-Diaminobenzidine
DAPI	Diamidino-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESAT-6	Early Secretory Antigenic Target of 6kDa

FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte-colony stimulating factor
GRO	Growth-related oncogene
HCL	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
IFN- $\gamma$	Interferon-Gamma
Ig-G	Immunoglobulin-G
IGRA	Interferon- $\gamma$ release assay
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kDA	Kilo Dalton
LAL	Lumulus Amebocyte Lysate
LAM	Lipoarabinomannan
LB	Luria-Broth media
LM	Lipomannan
LPS	Lippolysaccharide
mAb	Monoclonal antibody
MAC	Membrane Attack Complex
ManLAM	Mannose capped Lipoarabinomanann
<i>M. avium</i>	<i>Mycobacterium avium</i>
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
<i>M. bovis</i> BCG	<i>Mycobacterium bovis</i> BCG
MBL	Mannose binding lectin
MBP	Maltose binding protein



MDC	Macrophage derived chemokine
mg	Milligram
MR	Mannose receptor
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
<i>M. tb</i>	<i>Mycobacterium tuberculosis</i>
MHC	Major Histocompatibility Complexes
MIC	Minimum Inhibitory Concentration
ml	Millilitre
mRNA	Messenger RNA
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NF $\kappa$ B B cells	nuclear factor kappa-light-chain-enhancer of activated
NK cell	Natural killer Cell
nm	Nanometre
NO	Nitric Oxide
OD	Optical Density
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PI	Propidium Iodide
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PPD	Purified Protein Derivatives

PRR	Pattern Recognition Receptors
qPCR	quantitative Polymerase Chain Reaction
rfBC	recombinant fragment of conglutinin
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediates
ROIs	Reactive Oxygen Intermediates
rRNA	Ribosomal Ribonucleic Acid
RPM	Round per minute
RPMI	Complete Roswell Park Memorial Institute medium
SD	Standard Deviation
SEM	Standard Error of Mean
TGF- $\beta$	Transforming growth factor-beta
Th cells	T Helper Cells
TLR	Toll-like receptors
TNF- $\alpha$	Tumor Necrosis Factor-Alpha
tRNA	Transfer Ribonucleic Acid
WHO	World Health Organisation
Mg	Microgram
ml	Microliter
$\mu$ M	Micro Molar

## **Chapter 1 : Introduction**

## 1.1 Bovine tuberculosis

Bovine tuberculosis (BTB) is a contagious disease that occurs in livestock due to *Mycobacterium bovis*, which belongs to the *Mycobacterium tuberculosis* complex (MTC) and has economic impacts worldwide (Michel *et al.*, 2010; Muller *et al.*, 2013). Although a major host of *M. bovis* are cattle, the bacterium is not just restricted to bovidae species and can cause a disease in a wide variety of mammals including wild and domesticated animals such as cats, dogs, horses, sheep, goats, pigs etc, as well as humans due to its zoonotic potential (Barron *et al.*, 2015).

*M. bovis* infections occur in many countries across the globe. It is more commonly found in developing countries across the world specifically in Africa where a large number of cattle and wild mammal species (Fitzgerald and Kaneene, 2013). Due to the implementation of preventive and eradication programmes, the rate of BTB has greatly reduced in many developed countries including the United Kingdom, however a key problem identified in these countries is the presence of *M. bovis* in wild animals. The low-level prevalence of *M. bovis* in wild animals and its potential to spread to different mammalian species including domesticated and farm animals are some of the major challenges that are preventing the complete eradication of bovine tuberculosis (Schiller *et al.*, 2010).

An *M. bovis* infection in livestock primarily occurs due to the transmission of bacteria through the respiratory route, via inhalation of aerosol droplets containing the pathogen from an already infected host (Phillips *et al.*, 2003).

## 1.2 Epidemiology of bovine tuberculosis

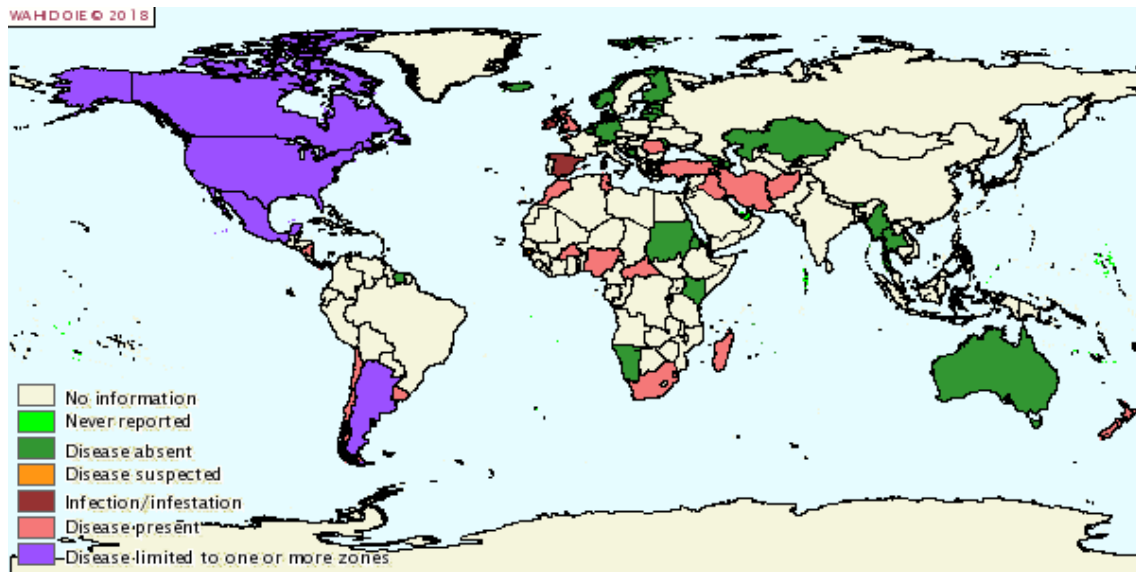
Although bovine tuberculosis is a disease with a substantial history, the epidemiology of this disease is still poorly understood. The occurrence of BTB incidences can vary between differing parts of the same country. Also, the difference in livestock management procedures in various parts of the world is another factor that may impact on the occurrence of BTB worldwide (Ayele *et al.*, 2004). Across many developed countries, BTB has largely been eradicated from cattle populations due to effective control and containment programs. However, the continuous presence of BTB in different wild mammals (such as badgers, white-tailed possums, deer etc) is a

matter of great concern as these wild organisms can act as reservoir hosts and a persistent source of infection for cattle, thus making the complete eradication of the disease extremely difficult (Byrne *et al.*, 2014; Kean *et al.*, 1999).

In most of the developing countries, BTB is still having a detrimental impact on the livestock sector with significant impacts on both cattle farming and public health due to the lack of implementation of proper control and eradication programs. During the last few decades the geographical distribution of BTB worldwide has changed significantly. Austria and North America have completely eradicated the disease or very close to achieving the free of the BTB. Still, a few developed countries such as New Zealand, parts of United States of America, Ireland and United Kingdom are facing challenges to control the *M. bovis* infection spread by wild life animals (Thoen *et al.*, 2008).

In developing countries BTB is still highly prevalent in herd populations, domestic animals and wildlife. The true epidemiological state of the disease is still not known since the majority of the cases of BTB remain unreported. Despite all of the above there is still enough evidence to indicate that BTB is on the rise in these countries particularly in Africa, Asia and Latin America (Cleaveland *et al.*, 2007; Etter *et al.*, 2006).

Worldwide Animal Health Information Database (WAHID) confirmed the *M. bovis* infection occurrence in 45 countries in 2016. The map below shows the worldwide distribution of BTB from data present at WAHID.



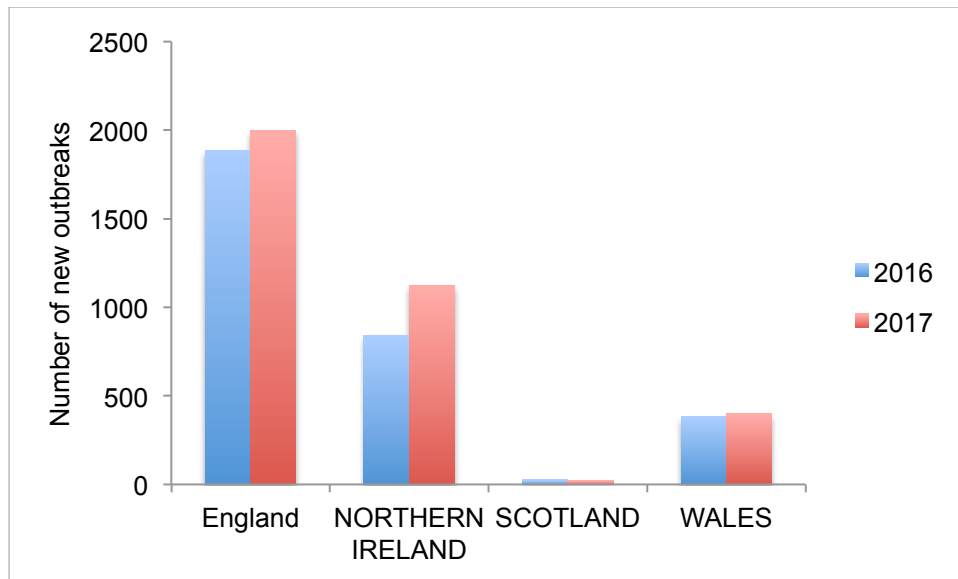
**Figure 1. 1: Worldwide incidence of *M. bovis* BCG infection.**

The data is for all the incidences of *M. bovis* infections recorded in the Worldwide Animal Health Information Database (WAHID) by World Organization for Animal Health (OIE) from January 2016 to December 2016.

### 1.3 Incidence of bovine tuberculosis (BTB) in the United Kingdom

Bovine tuberculosis is one of the major disease effecting herd populations in the United Kingdom. Data shows that in 1979 only 0.01 percent of the total cattle population was positive when tested for BTB. However, the disease has spread significantly to all parts of the United Kingdom over the last three decades. According to the draft for the eradication strategy for England published in April 2014, it was proposed that the country aims to eradicate BTB completely from its cattle population by 2038. One of the main strategies is to divide the country in to three risk areas on the basis of BTB prevalence; High Risk Area (HRA) with high levels of BTB infection present in cattle and also a significant reservoir of BTB in wild life (badgers); Low Risk Area (LRA) with low level of BTB infection and no significant reservoir of BTB in wild life ; Edge Area characteristics are variable levels of BTB infection, higher than LRA but lower than HRA and also the role of wild life in spreading diseases is uncertain (DEFRA, 2016). As a part of the surveillance and control program, almost 6.2 million BTB tests were performed in England in 2014 and more than 26,000 cattle were slaughtered to control the spread across the herd population (DEFRA, 2016).

Data on BTB incidences in the United Kingdom released by DEFRA in September 2017 shows that in 2016 there were 3753 new herds with BTB incidents in England which was relatively similar in 2015 cases. Since 2011, the total number of infected herds has stabilized in between 37,00 and 39,00 (DEFRA, 2017). In 2016 the total number of infected herds was slightly lower than 2015, HRA and LRA reported 3229 and 137 respectively infected herds slightly less compared to 2015, in 2015 there were 3401 (HRA) and 156 (LRA). However, in edge areas which is the buffer zone in between HRA and LRA contains lower BTB than in the HRA but higher BTB as compared to LRA, the number of BTB incidents were reported in 387 herds in 2016, more than 2015, which had reported 339 infected herds (DEFRA, 2017).



**Figure.1.2: Comparison of new incidences of *M. bovis* infection from 2016-17.** The data shows all the new incidences of *M. bovis* in cattles in the UK, recorded by WAHID on behalf of the World Organization for Animal Health (OIE) from January-June 2016 and January -June 2017.

The current surveillance and control system for BTB in United Kingdom uses the routine tuberculin skin test IFN- $\gamma$  release assay to detect *M. bovis* infections in animals and culling of the infected populations accompanied by post-mortem surveillance. This strategy works well for the cattle herds and domesticated animals. Parts of the United Kingdom have been successful in reducing the level of BTB and preventing the establishment of the disease in their herd. Badgers have been identified as one of the most important wildlife reservoir hosts for BTB in the United Kingdom for transmission of BTB to cattle (Abernethy *et al.*, 2013). A reservoir is usually an animal or a plant, inside of which a pathogen survives, often (though not always) without causing disease for the reservoir itself. *Mycobacterium bovis* can also infect and cause disease in badgers.

#### 1.4 The role of badgers in the epidemiology of bovine tuberculosis

Although cattle are the most common hosts for *M. bovis*, other domestic and wild animals may act as a reservoir of infection (Corner, 2006). This causes difficulty in the eradication of BTB from cattle due to the continuous exposure/contact from these animals. There are several badgers species in different countries that act as reservoirs of *M. bovis* infection, those include European badgers (*Meles meles*) in the UK and Republic of Ireland, the European wild boar present in Spain (*Sus scrofa scrofa*), New



Zealand have the brush tail possum (*Trichosurus vulperula*), in north America the white tailed deer (*Odocoileus virginianus*), in South Africa buffalo (*Syncerus caffer*), and the lechwe antelope (*Kobus leche*) in Zambia (More and Good, 2006; Delahay *et al.*, 2007; Fitzgerald and Kaneene, 2013).

In 1971, the first badger reported with *M. bovis* infection was in the southwest region of England (Murhead and Burns, 1974), however these findings were controversial at that time. Most recently *M. bovis* infection has also been reported in badgers in Spain (Balseiro *et al.*, 2013) and France (Payne *et al.*, 2013). It has been reported that BTB is endemic in the badges across some parts of United Kingdom where they are actively contributing to the spread of infection in cattle (Eves, 1999; Donnelly *et al.*, 2006). This is now established in the United Kingdom that badgers can be infected by *M. bovis* According to the research study, badgers infected with *M. bovis* have been reported 1.6% to 37.2% in low prevalence of bovine BTB areas to high prevalence of bovine TB areas (Ni Bhuachalla *et al.*, 2014).

*M. bovis* infection can be transmitted to the cattle by infected badgers either by infectious aerosol routes or by swallowing of contaminated feed with badger faeces (Gallagher *et al.*, 1976). The risk factors to transmit the disease from infected badgers to cattle includes the infection presence across the badger community, the phase of disease, the method of exposure in cattle and level of excretion from infected badgers (Gallagher and Clifton, 2000).

In 1950, the eradication scheme for BTB from UK was introduced to make the country free of BTB (DEFRA, 2014). To achieve the goals of the eradication scheme, the regular testing of the cattle was implemented, and this resulted in reduced incidences of cattle to cattle transmission. Despite that, the complete eradication goals have not been attained and for that mainly the lack of available diagnostics tests were blamed. When badgers were established as the host of *M. bovis*, different options have been reviewed to control the BTB in badger populations (Cheeseman *et al.*, 1981). The main objective of this strategy was to minimize the transmission of *M. bovis* infection from badgers to cattle by implementing different strategies. The strategies comprised of culling of badgers, to keep two species (badgers and cattle) separate by improved biosecurity measures and vaccination. The badgers are listed as a legally protected species in the United Kingdom (Protection of Badgers Act, 1992).

However, there are exemptions in the law for culling of badgers in order to control the BTB infection in cattle (Roy and Milton, 2004). The main purpose of culling of the badger is to reduce the population of badgers so as to minimize the possibility of transference of *M. bovis* to the cattle. Culling can be selective or non-selective. In selective culling, infected populations or individuals can be culled and in non-selective culling involves the infected badger population on the speculation basis with the thought to decrease in badger numbers will reduce the chances of infection to the cattle's (Smith *et al.*, 2016).

The badger contribution in the transference of BTB in United Kingdom has been estimated from different research studies of culling and badger eradication operations performed over the previous 30 years. A non-specific badger culling trial was performed in between 1998 and 2005, which had a significant negative effect on the UK government stance on badger culling (Bourne *et al.*, 2006). In 2011, farmers have been given permission to reduce the badger's populations in highly affected areas in aiming to stop the disease transmission to cattle. Licenses were issued to farmer by Natural England under the Protection of Badgers Act 1992 for badger cullings (DEFRA, 2014).

### 1.5 Characteristics of *M. bovis*

*M. bovis* causes tuberculosis in wild and domestic animals. *M. bovis* belongs to genus *Mycobacterium* and family *Mycobacteriaceae*. *Mycobacteria* are non-motile, obligate aerobic and Gram-positive bacilli (Mostowy *et al.*, 2005). *M. bovis* is part of the *Mycobacterium tuberculosis* complex (MTC) which includes; *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium lepra*, *Mycobacterium marinum*, *Mycobacterium ulcerans*, *Mycobacterium avium*, *Mycobacterium microti*, *Mycobacterium canetti* and *Mycobacterium smegmatis* (Smith *et al.*, 2006).

*M. bovis* is a slow-growing (16-20 hours generation time), aerobic intracellular pathogen which commonly resides inside the phagocytic cells. *M. bovis* is a straight or slightly curved rod, Gram-positive acid-fast bacterium with dimensions of 1-4  $\mu\text{m}$  in length and 0.3-0.6  $\mu\text{m}$  wide (Barry *et al.*, 1998). The genome of *M. bovis* is 4,345,492 base pair long and is organized in a single circular chromosome. The

genome comprises of approximately of 3952 proteins encoded genes. The *M. bovis* genome has 99.52% similarity to *Mycobacterium tuberculosis* (Garnier *et al.*, 2003).

### **1.6 Transmission of *M. bovis***

*M. bovis* infections can occur in a variety of animals resulting in BTB and the infected hosts can be categorized as reservoir hosts. The reservoir hosts act as a stable host and can be a persistent presence of the tuberculosis within local populations. The reservoir hosts do not maintain a persistent *M. bovis* infection and need other species for persistent presence of disease within a population and may transmit the infection between their members for a certain period of time. Wild mammals are believed to be the reservoir host for *M. bovis* in many countries of the world and are believed to be responsible for the continued presence of bovine tuberculosis in domestic animal and cattle herds (Baron *et al.*, 2015; Humblet *et al.*, 2009).

There are several ways through which *M. bovis* can spread and cause BTB in cattle and other organisms. The primary route of infection of *M. bovis* infection is via the respiratory route, however, the bacteria can also cause infection via other routes including oral, congenital, breaks in the skin or wounds and occupational acquisition of the disease. The consumption of milk from an infected cow by calves is also a source of transmission of BTB (Skuce *et al.*, 2012).

The transmission of *M. bovis* among cattle rely on several aspects such as dose of infectious inoculum, route of infection, duration of contact and the immune status of the host. The transmission of bacteria by respiratory route between cattle may also require the production of very fine aerosol droplets for bacterial shedding which are only produced under certain conditions when the active disease is established in the primary host. As the disease becomes more established in the primary host the chances of transmission to other cattle enhances due to the large amount of pathogen shed (Skuce *et al.*, 2011).

### **1.7 Pathogenesis of bovine tuberculosis**

BTB presents mainly as lesions in the upper respiratory lymph nodes of the lung and thorax. Alveolar macrophages are the first to interact with the bacteria when infection

occurs through its main route via, the respiratory tract (Cassidy, 2006). Although the aetiology and immune response to *M. bovis* is similar to *M. tuberculosis* in humans, the pathology of the disease needs to be further investigated, in particular the molecular basis of the interaction *Mycobacterium*-macrophage, which could lead to selective and effective therapeutic development (Pollock and Neill, 2002).

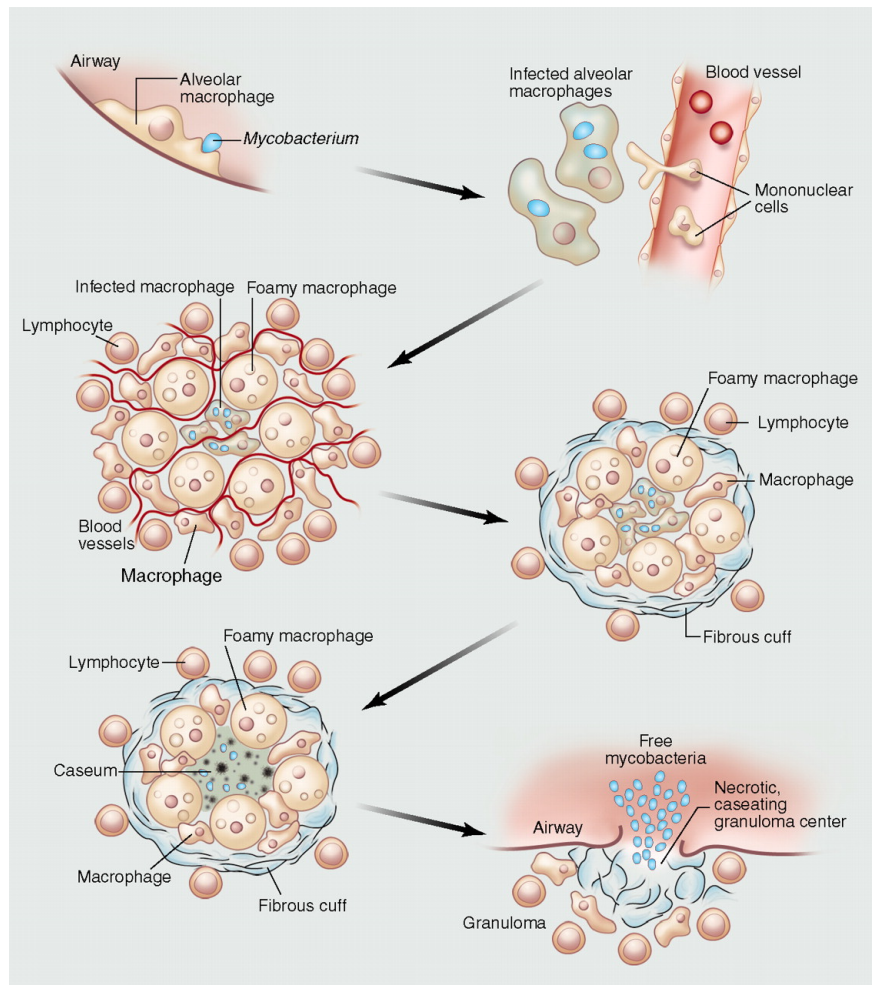
In BTB establishment of lesions depends on the route of transmission of the pathogen. The most common route of bacterial entry into the host is by inhalation that causes lesions of the upper respiratory tract mucosa, nasopharynx, retropharyngeal lymph nodes, lower respiratory tract and lungs (Neill *et al.*, 2001) and ingestion of bacteria causes lesions of the mesenteric lymph nodes and gastrointestinal wall. Trans-placental and genital transmission of mycobacterial infections is very rare due to the active eradication programs of bovine TB in most of countries (Menzies and Neill, 2000).

After entry into the host, macrophages, neutrophils and dendritic cells are recruited at the site of infection (Domingo *et al.*, 2014). Macrophages may be able to destroy the mycobacteria if bacilli are lower in numbers. However, mycobacteria can evade immune responses and survive and replicate intracellularly (Dannenberg, 2001). The macrophages engulf the mycobacteria and migrate to regional lymph nodes, where the protective Th1 response is induced ending in the formation of granulomas (Pollock and Neill, 2002).

In some animals, strong cell mediated immunity prevents the formation and extensions of the lesions to other organs, but the presence of the immune response in alveolar spaces and pulmonary airways is usually unable to control the spread of infection. As a result, small lesions developed into the larger ones over time, causing chronic tuberculosis in the lungs. Chronic lesions in the lungs are identified as caseous necrosis and finally lesion formation occurring inside the affected lobe. Similarly, lymph nodes are chronically affected and become enlarged in size covered by granulomatous caseous material surrounded by fibrous tissues (Domingo *et al.*, 2014).

### 1.7.1 Granuloma structure and function

Granuloma formation in the lungs is the result of mycobacterial infection. This is the main pathology of tuberculosis in humans and animals. Granulomas consists of different immune cells such as tissue lymphocytes, macrophages, natural killer cells and neutrophils, these cells contribute in the formation and function of the granuloma. In human tuberculosis alveolar macrophages induce the pro-inflammatory response after engulfing mycobacteria and this initiates a T-cell response by attracting CD4<sup>+</sup> T-cells, which lead to the recruitment of fibroblasts, CD8<sup>+</sup> T-cells and B-cells attracting mononuclear cells from neighboring blood vessels. Different types of cytokines and chemokines such as TNF-  $\alpha$  and INF- $\gamma$  are released by infected macrophages that play a significant role in the formation of granuloma, where as IL-10 is a negative regulator of granuloma formation (Hogan *et al.*, 2001). These immune cells form building blocks for the granuloma (or tubercle), which is a circumscribed yellowish granulomatous inflammatory nodule approximately 2–20 mm in diameter. The granuloma has a necrotic, caseous center surrounded by neutrophils, macrophages and langhans-type multinucleated giant cells (Neill *et al.*, 2001).



**Figure. 1.3: The life cycle of *Mycobacterium* and granuloma formation.** Infection starts after inhalation of droplets that contain bacilli and are phagocytized by macrophages. Pro-inflammatory responses promote recruitment of other immune cells; more macrophages, dendritic cells and lymphocytes towards the infected site and form the granuloma. The macrophages in the granuloma developed to form epithelial cells, multinucleate giant cells and cells filled with lipid droplets. The granuloma can be matured by the formation of the extracellular matrix material outside the macrophages layer (Russel *et al.*, 2010).

Granuloma formation helps in preventing the spread of mycobacteria to other organs of the host, and also granuloma represents a delicate host-pathogen stand-off that will not harm the host and does not kill the mycobacteria called latent tuberculosis infection (LTBI) (Sundaramurthy and Pieters, 2007).

## 1.8 Zoonotic impact of bovine Tuberculosis

Bovine tuberculosis can infect humans through the inhalation of air droplets with bacteria after an infected animal or person coughs or sneezes, by consuming raw dairy products such as unpasteurised milk or uncooked meat from infected animals. Cattle owners, TB testers and dairy product handlers are those who have direct contact with infected animals, are at a higher risk of bovine TB (Cosivi *et al.*, 1998). Zoonotic tuberculosis is a public health concern in developing countries. High similarities between *Mycobacterium tuberculosis* and *Mycobacterium bovis* infections can make it difficult to estimate how much of the population is infected with anything other than *Mycobacterium tuberculosis* (De la Rúa-Domenech, 2006).

Improved hygiene levels in the dairy industry and pasteurisation of milk in developed countries has drastically dropped down the cases of human infection by *M. bovis*. However, migration from developing countries with a less developed meat and dairy industry increases the level of BTB (Cotter *et al.*, 1996). It is known that in 1980 and 1991 the level of tuberculosis infection increased in San Diego, because of the migration of farmers from South America and Mexico having a higher rate of cattle infected by *M. bovis* (Dankner *et al.*, 1993).

*M. bovis* reportedly caused a significant number of tuberculosis cases in children and farmers in rural areas (Schmiedel, 1968). 5 to 20% of human TB was reported in developed countries before 1960s (Hardie and Watson, 1992). In countries where BTB is common, and milk is rarely pasteurized. This risk of acquiring BTB in humans is low in the UK (Torgerson and Torgerson, 2010).

## 1.9 Symptoms of bovine tuberculosis

The symptoms of BTB usually takes months to develop in cattle. Once the bacteria start actively dividing, the disease may develop rapidly and is accompanied by different symptoms that are commonly found across all species of host animals although there can be certain species variations. Some of the symptoms associated with BTB in animals include progressive weakness, anorexia, lack of appetite and fluctuating fever. Infection of the pulmonary system by the bacteria causes a moist

cough in the animals that is worse during in the morning or during cold weather conditions, causing difficulty in breathing (Menzies and Neill, 2000).

In some animals the lymph nodes in the retropharyngeal area may become enlarged during BTB. The enlarged lymph nodes can rupture and can also block blood vessels or airways. If the bacterial infection is obtained through oral route, diarrhea or constipation may be observed in the infected animal. Skin infections in certain domesticated animals like cats have also been observed in the form of soft swelling or flat ulcers mostly visible on the head, neck or shoulders. Some animals such as brush tailed possums, become disoriented during the final stages of the disease and appear to be extremely weak (Menzies and Neill, 2000).

### **1.10 Diagnosis of bovine tuberculosis**

Diagnosis of BTB in cattle and other animals can be done either by direct detection of the bacterial presence inside the host or through indirect methods. The direct detection mainly involves a post-mortem examination of animals. This includes the use of microscopy to confirm the infection in animals previously testing positive for BTB by a pre-mortem test. Since most of the cattle and other infected hosts do not necessarily show the symptoms of the disease for a considerable period of time, the direct test is not very effective in controlling BTB.

Indirect tests make use of the different indicators of *M. bovis* infection in live animals and are widely used for the diagnosis of BTB. Indirect diagnostic tests make use of immunological markers produced in response to *M. bovis* infection. These tests consist mainly of two types. First type of test includes intradermal tuberculin skin and INF- $\gamma$  release assay (IGRA) test, which are established because of cell-mediated immunity to *M. bovis*. The second type of diagnostic test detects the antibodies responses in susceptible hosts (Wood *et al.*, 1992).

#### **1.10.1 Tuberculin skin test (TST)**

Intradermal tuberculin test is the most common type of diagnostic test currently used and involves the injection of a small amount of PPD (purified protein derivatives of *M. bovis*) under the skin of the caudal fold (CFT) or neck (CIT) of an animal. The



skin on the neck is more sensitive to a tuberculin-related hypersensitivity reaction as compared to skin of caudal fold. The immune system reacts to the tuberculin resulting in a localized allergic reaction which is measured after 48 to 72 hours to predict the presence of *M. bovis* (Schiller *et al.*, 2010).

### **1.10.2 Interferon gamma (INF- $\gamma$ ) release assay (IGRA) test**

Interferon- $\gamma$  (Interferon gamma) release is applied as an additional confirmatory test of cattle to the TST or alongside TST to increase diagnostic sensitivity. *M. bovis* antigens ESAT-6 and CFP-10 induces the secretion of INF- $\gamma$  in host. *M. bovis* BCG vaccine strain does not contain these proteins, so the assay is more precise than the TST (Ramos *et al.*, 2015).

### **1.10.3 Post mortem diagnosis of *Mycobacterium bovis* infection**

Post-mortem diagnosis of BTB is another important cost-effective diagnostic technique, which observes lesions after slaughter of the animal. The identification of infection in post-mortem diagnosis, initiates the investigation of the disease in herds and the origin of infection (Whipple *et al.*, 1996; Olea-Popelka *et al.*, 2008).

### **1.10.4 Histopathologic examination**

Diagnosis of BTB can be done by histopathology or microscopic confirmation of mycobacteria. Tissues or smears from the clinical samples can be directly stained with Ziehl/ Neelsen stain to diagnose the infection. Although pathological changes can be a good indication of *M. bovis* infection they are not always definitive. Different confirmatory tests including bacteriology and molecular methods such as PCR accompanied by histopathology can be used to establish the presence of an actual infection (Ramos *et al.*, 2015).

## **1.11 Treatment and prevention of BTB**

It is extremely difficult to treat tuberculosis infection in animals, so they are culled rather than treating them. In the United States and many other countries, it is not permitted to treat TB in animals (Thoen *et al.*, 2008). If the infected animal is treated it can take as long as 9 months due to the resistant nature of *M. bovis* and a

combination of different antibiotics at different levels may be required. One of the main reasons for the lack of any treatment regimens is the expensive nature of *M. bovis* treatment which makes it economically unfeasible to treat the disease (DEFRA, 2016).

There is currently no effective vaccine available to prevent against BTB. The lack of effective vaccine makes the use of preventative measures extremely important for the prevention and transmission of disease (Waters *et al.*, 2012). It is very difficult to distinguish between vaccinated cattle and infected cattle by performing tuberculin skin tests. United States and other countries with eradication programs have stopped the use of vaccines (Skinner *et al.*, 2003).

Pre-movement and post-movement testing for *M. bovis*, in herd populations is essential to decrease the transmission of *M. bovis* between herds and from one area to another. The herd animals should be screened on regular basis for the presence of *M. bovis* using the above diagnostic tests. Cattle who test positive for *M. bovis* are culled to prevent any further transmission.

Furthermore, to minimize the risk of cattle-to-cattle spread of *M. bovis*, lower densities of cattle tend to be kept in herds. Double fencing is also necessary to stop the nose-to-nose spread of BTB to neighbouring farms. Double fencing also prevents the contact of herd animals with wildlife. Pasteurization of milk is also important to prevent the possible infection to humans, since *M. bovis* cannot survive at high temperatures (Tebug *et al.*, 2014).

Other control measures include basic herd hygiene, restricting movement of an infected animal and the maintenance of food and water hygiene has been found to reduce the risks of disease transmission (Phillips *et al.*, 2003). In domestic herds, culling is a successful tool of controlling the infection in livestock operations. The effects of depopulation can be damaging both financially and emotionally to farmers. Countries using other strategies alternatively to the depopulation of herds, like in the United States, the U.S. Department of Agriculture asks regulatory authorities to perform herd-specific tests and slaughter programs for individual livestock operations (USDA, January 2005). In Europe and New Zealand, the regular testing and removal

of animals that have tested positive for BTB from the herd, results in no movement of animals other than to slaughter, until the herd has tested negative (Cousins, 2001).

### 1.12 Biomarkers for Bovine tuberculosis

The practice of “test and slaughter” programmes in most of developed countries seems to control bovine tuberculosis. In developing countries with a high number of BTB cases, it is not feasible to apply the “test and slaughter” policy due to economic limitations. In such circumstances, there is more need to improve the vaccination and diagnostic strategies to control the disease. Blood or serum based diagnostic techniques can be used to diagnose the infection at various stages in the host to prevent the spread of disease by isolating the infected animals from herds.

Currently, the tuberculin skin test and IFN- $\gamma$  tests are used as a diagnostic tool in BTB (Ramos *et al.*, 2015; Gormley *et al.*, 2006). IGRA in blood after specific antigen activation has been a helpful method for the diagnosis of infections in cattle (Vordermeier *et al.*, 2011; Lim *et al.*, 2012).

BTB infection commonly occurs in macrophages, where the pathogen can survive, grow and spread to other sites of the body (Thacker *et al.*, 2007). Cell-mediated immunity (CMI) plays a vital role in the host’s immune response. It has been shown that IFN- $\gamma$  plays a vital role in CMI. Studies have shown that mice deficient in IFN- $\gamma$  production failed to prevent the BTB infection in the mouse (Cooper *et al.*, 1993; Flynn *et al.*, 1993).

Proteomics offer a new way of early detection of *M. bovis* infection by discovering novel biomarkers. Isobaric tag for relative and absolute quantification (iTRAQ) is a type of proteomics technique, a liquid chromatography and tandem mass spectrometry technique that permits diagnosis and quantification of peptides between multiple sample groups (Seth *et al.*, 2009). It has been studied that vitamin DBP is up regulated during early bovine TB infection. There are six other proteins found in infected cattle are fetuin, serine proteinase inhibitor (alpha-1-antitrypsin, AAT), complement C3, alpha-1 acid glycoprotein and alpha-1 beta glycoprotein (Seth *et al.*, 2009).

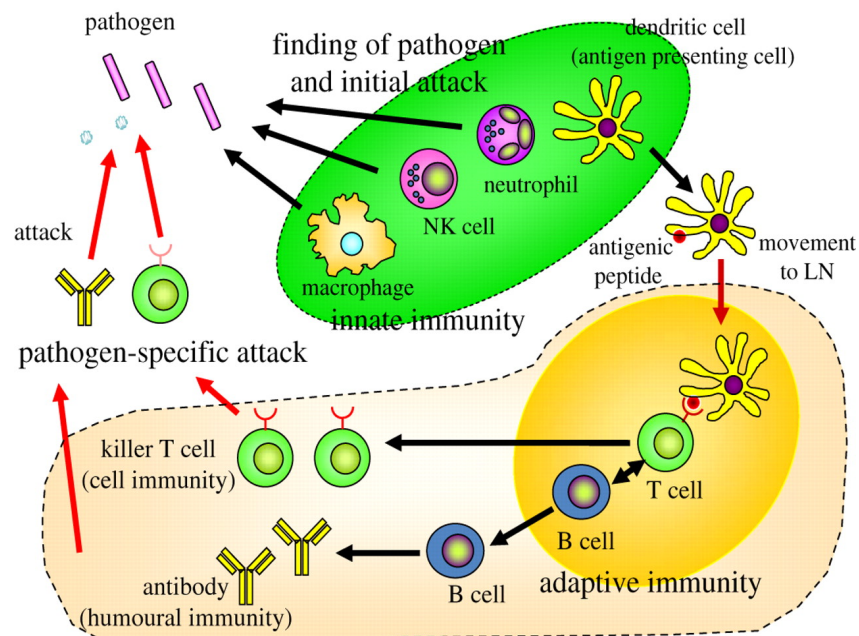
To prevent the bovine TB in the United Kingdom involve statutory screening of the cattle herds using the single intradermal comparative tuberculin test (SICCT) and further culling and movement restrictions applied if test-positive cattle are detected. In addition, abattoir surveillance is carried out by specialist meat inspectors on all SICCT test reactor cattle looking for evidence of suspect tuberculous lesions in defined organs and body sites. Inspection should be carried out on all cattles those been slaughtered for human food chain (Bermingham *et al.*, 2014).

Although there are preventive measures in place to avoid the spreading of bovine TB, it is likely that control and eradication will require additional control strategies. A potentially powerful approach would be to study the genetic variation in host resistance to bovine TB. Previous studies suggested between-host genetic variation in resistance to TB exists in many species, including humans, mice, deer and cattle (Allen *et al.*, 2010). Quantitative genetic studies in herds in United Kingdom and Republic of Ireland observed significant heritability for host resistance existence in dairy cattle herds (Bermingham *et al.*, 2009, 2010; Brotherstone *et al.*, 2010). Recently, several studies have begun to address the identification of genetic loci associated with bovine TB resistance. Polymorphisms in candidate genes, *SLC11A1* in African Zebu cattle (Kadarmideen *et al.*, 2011) and (TLR1) in Chinese Holsteins (Sun *et al.*, 2012) have been significantly associated with bovine TB infection. Two genomic regions identified by the microsatellite markers INRA111 and BMS2753 were also linked with SICCT reactor status in United Kingdom cattle (Driscoll *et al.*, 2011). Finally, a recent genome-wide association study (GWAS) in Holsteins in Republic of Ireland identified a genomic region on *Bos taurus* chromosome 22 containing the taurine transporter gene *SLC6A6*, which was suggestively associated with bTB resistance, although it only explained a small proportion of the total variance due to genetic factors (Finlay *et al.*, 2012).

Thus, host genetic control of resistance to bTB in cattle is likely to be complex and involve many loci of varying effect. Furthermore, each study to date has had limitations in terms of the number of animals studied and the genomic tools deployed.

### 1.13 Innate immunity

Host immunity is made up of the innate and adaptive immune response. The adaptive immune response is divided into humoral immunity and cellular immunity. Humoral immune response plays an important role in eliminating pathogens present in the body by producing specific antibodies by B-cells against pathogens (Akira, 2011). Cellular immunity takes part in the eradication of microbes and cancerous cells by the help of killer T cells and B cells. These cells have distinctive T-cell receptors (TCRs) and B-cell receptors (BCRs), and identified a wide range of different antigens (Akira, 2011).



**Figure. 1.4: Innate and adaptive immunity interaction:** After entering of microbes in the host, innate immune system is initiated and plays a vital role as a first line of defence in the body against the pathogen. Dendritic cells act as antigen-presenting cells to T cells in lymph nodes from infected tissues. Adaptive immune system is activated after the innate immune response, antibody production and killer T cells activated to attack the pathogens directly (Akira, 2011).

The innate immune response is the first line of defence against pathogens. Innate immunity is mediated by phagocytes those are leukocytes, dendritic cells, and macrophages engulf and kill the pathogens and take part in the presentation of antigenic peptides to T cells (Akira, 2011).

### 1.14 Immune response against *M. bovis*

The activation of innate immune responses against pathogens is very critical at early stages of pathogen entry into the host, which is recognition of the microbes by macrophages (Pollock and Neill, 2002). Macrophages provide hostile environment to mycobacteria, but also contributes in the prevention and eradication of pathogens (Aldwell *et al.*, 1996).

Other immune cells may be involved in the response of initial tuberculosis infection. Natural killer (NK) cells play a major role in human tuberculosis. NK cells can produce IFN- $\gamma$  and destroy mycobacteria-infected target cells (Yoneda & Ellner, 1998). These cells are defined as large granular cytotoxic type of lymphocytes. Their unique role is to identify and destroy the harmful cells which do not have MHC I markers, as these harmful cells cannot be detected by any other immune cells. So they were named as NK cells due to the ability that NK cells do not require activation to kill cells those donot have MHC I markers. It has been studied that NK cells increase the synthesis of important cytokines such as IFN- $\gamma$ , IL-12, IL-15 and IL-18 by macrophages *in vitro* (Kanakaraj *et al.*, 1999). It has been shown that NK cells are activated in the early stages of tuberculosis. Higher number of NK cells found in the lungs of tuberculosis infected mice after 21 days of infection (Junquera-Kipnis *et al.*, 2003).

Neutrophils are known as the first leucocytes to respond in the host's defence against invading pathogens, killing the pathogens by phagocytosis and intracellular killing via oxidative /non-oxidative. It has been reported that neutrophils are among the earliest immune cells helping in the formation of granuloma in BTB (Cassidy *et al.*, 1998). A similar role in human tuberculosis has also been identified (Guirado and Schlesinger, 2013). IL-17, IL-23 released by Th-17 cells and IL-18 by macrophages are important cytokines that attracts neutrophils in the tuberculosis infection and contribute to granuloma formation (Guglani and Khader, 2010).

Despite mechanisms of the innate immune system, mycobacteria successfully infect and persist in the macrophage. In human tuberculosis, macrophages recognise the mycobacterium through the interaction of pathogen-associated molecular patterns (PAMPS) with cell surface pattern recognition receptor proteins (PRRs), such as the

Toll-like receptors (TLRs). These PAMPs include bacterial lipopolysaccharides, peptidoglycan, lipoprotein, nucleic acid and mycobacterial lipoarabinomannan and mannose-capped lipoarabinomannan.

In the macrophages, mycobacteria produced either secreted antigens or break down components that are presented to T-cells. T-cells act as antigen presenting cells to initiate the immune response. In adaptive immunity, after the antigen is processed and recognised by the antigen presenting cells (APCs), the adaptive immune system creates immune cells designed to attack the antigen and effectively eliminate the pathogen from the host. It has been reported that cattle infected with *M. bovis*, have shown immune responses involved within this infection (Ritacco *et al.*, 1991). This immune response in cattle against *M. bovis* is identical to the immune response identified for human tuberculosis (Lenzini *et al.*, 1977).

Adaptive immunity starts in the lymphoid organs. In the bloodstream, T-cells and B-cells uniformly circulate to hunt for particular antigens. In tuberculosis infection, activation of the adaptive immune response is slow in comparison to other pathogens, this slower immune response helps bacterial multiplication inside the lungs. In mice the adaptive immune response activated after 10-12 days of tuberculosis infection (Wolf *et al.*, 2008). In the early stages of the tuberculosis infection, the mycobacteria hide inside the phagosome and stop dendritic cells and macrophages to present the antigen to T-cells. The prevention of presentation of antigen to T-cells provides enough time to bacilli for more multiplication in the lungs. This increase in bacterial population reduces the effects of an adaptive immune response (Wolf *et al.*, 2008). In the result of tuberculosis infection, Th1 cells produced high level of CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma/\delta$  T-cells. In the infected cattle, the changes in the circulating T-cells demonstrate the progression of infection with the time. It has been seen that  $\gamma/\delta$  T-cells had involvement in the initial immune response followed by CD4 and then CD8 T-cells (Pollock *et al.*, 1996).

### **1.15 Role of complement system in bovine tuberculosis**

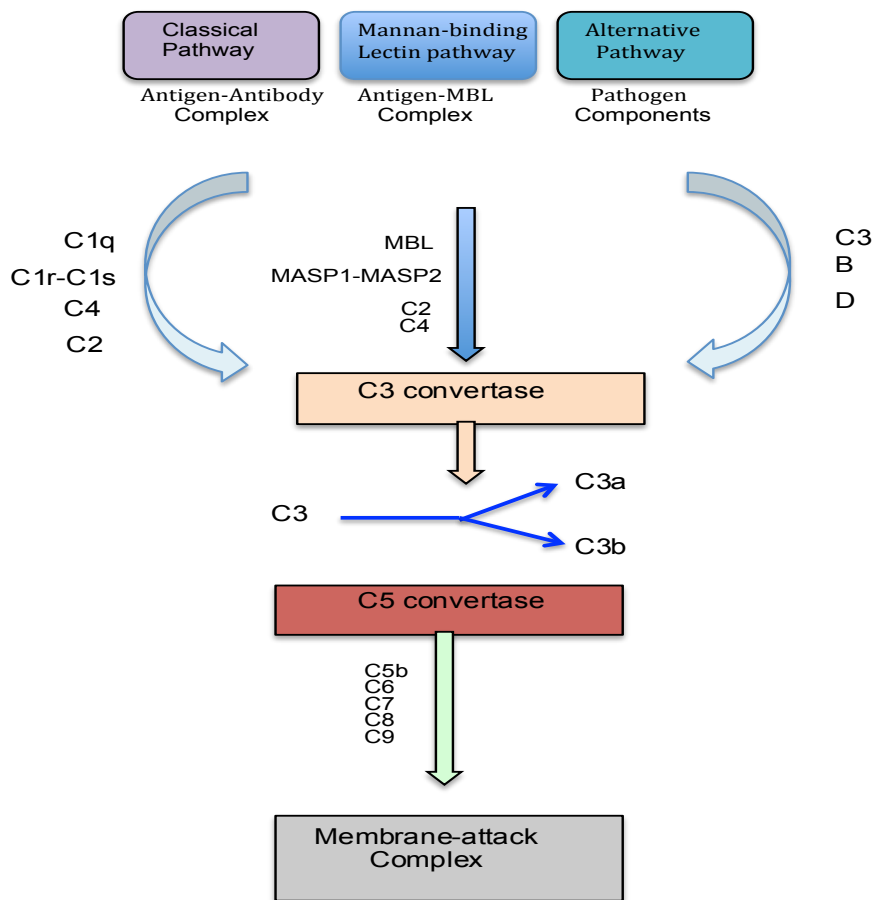
Complement system is comprised of 40 proteins, which are a major component of the innate immune system. Complement is activated by classical, alternative and lectin pathways (Gadjeva *et al.*, 2001). The classical pathway is initiated when antibody

recognise antigens on the surface of pathogens and these antibody and antigen complexes binds to the C1q proteins, whilst mannose-binding lectin (MBL) or ficolins bound to the complement activator leads to the activation of lectin pathway (Matsushita *et al.*, 2000; Matsushita and Fujita, 1992). A wide range of targets triggers the alternative pathway, it does not require specific initiator component such as C1q or MBL. Alternative pathway is usually triggered when C3b protein binds to the pathogen (Matsushita *et al.*, 2000).

Mycobacterium resides inside the macrophages and has ability to survive and multiply in the host immune cell. In BTB, *M. bovis* influence complement activation through its cell surface or secreted proteins (Carroll, 2009). *M. bovis* and *M. tuberculosis* have similar structure and *M. tuberculosis* invades and multiplies as an intracellular pathogen in the host immune cells therefore evading complement (Carroll *et al.*, 2009).

C3 convertase is the main product of any complement pathway, allowing the cleavage of C3 into C3a and C3b. C3b is deposited on microbial surfaces. Deposition of C3b can act as an opsonin on the surface of microbes and as well as other immune regulatory functions. The C3 convertase cleave the C3 protein into C3b and C3a components. C3a is an anaphylatoxin that activated the local inflammation where as C3b covalently bound non-selectively to surfaces of the pathogens. C3b acts as opsonin and enhance the clearance of the pathogens by immune cells and assists in cell lysis (Lachmann and Hughes, 1984; Pangburn and Muller, 1984). C3b also interacts with factor B and form C3bB complex. The reaction conducted by C4b2a in the classical and lectin pathways, is intensified by the alternative pathway, which generates more C3b and initiate the terminal sequence, common to all pathways. C3a and C5a-fluid phase anaphylatoxins, and iC3b and C3dg-cell-bound opsonins, facilitate phagocytosis (Pangburn and Muller, 1984).





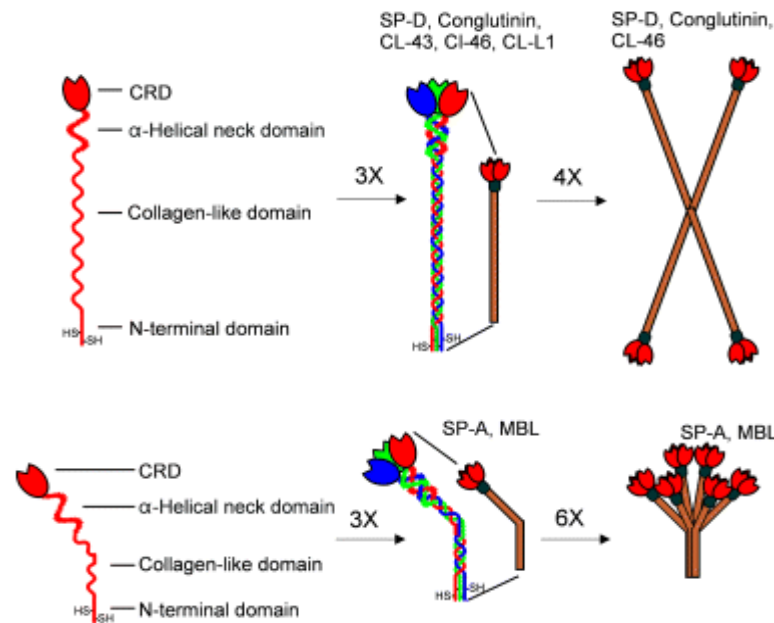
**Figure 1.5: Schematic of complement pathways.** In the early response of the immune system, classical, lectin or alternative pathways identify pathogens and activate complement reaction to destroy pathogens. After the activation of any complement pathways, this results in the synthesis of C3 convertase that initiates cleavage of C3 into C3a and C3b, producing C5 convertase. C5 convertase further initiates cleavage of C5 into C5a and C5b. C3a and C5a recruits immune cells such as basophils, eosinophil, dendritic cells and macrophages to the infectious site. Formation of the membrane attack complex C5b-9 is responsible for lysis of bacterial cells (Lachmann and Hughes, 1984; Pangburn and Muller, 1984).

## 1.16 Collectins and their roles in innate immunity

Collectins are soluble innate immune molecules, also called C-type lectins that play a vital role during the early stage of infection as well as other immune functions. Collectins prevent infection by various mechanisms, including opsonisation, growth inhibition, neutralization (Sano and Kuroki, 2005; Kishore *et al.*, 2006; Haagsman *et al.*, 2008). Well known members of the collectin family are surfactant protein A (SP-A) and D (SP-D), bovine conglutinin (Bc), mannan-binding lectin (MBL), collectin placenta 1 (CL-P1), collectin liver 1 (CL-L1), collectin of 43 kDa (CL-43) and collectin of 46 kDa (CL-46), all of which have a common structure. Their monomeric subunits are composed of N-terminal attached via  $\alpha$ -helical neck regions to C-terminal, also known as carbon recognition domains (CRD) (Wang *et al.*, 1995; Hansen and Holmskov, 2002). These monomers are stabilised by covalent disulphide bridges as well as non-covalent interaction that form a trimer confirmation by binding cysteine residues in N-terminals or in collagen domains (van *et al.*, 2004).

Conglutinin has close structural similarities to SP-D, being able to form multivalent cruciform structures (tetramers of trimers) containing in total 12 CRD regions that can bind to microbial surfaces in the presence of  $\text{Ca}^{2+}$  (Hansen *et al.*, 2002). Conglutinin is secreted largely by the liver and is found in bovine serum at a concentration of 12  $\mu\text{g}/\text{ml}$  (Holmskov *et al.*, 1998). In contrast, SP-D is primarily found in the lungs at a concentration of 0.1–0.9  $\mu\text{g}/\text{ml}$  and is secreted by alveolar type II cells and Clara cells (Kishore *et al.*, 2006). Conglutinin is thought to have evolved in the *Bovidae* from a gene duplication event of an ancestral SP-D gene and is located on chromosome 28 in *B. taurus* (analogous to chromosome 10 in *Homo sapiens*), proximal to the bovine SP-D gene (Gallagher *et al.*, 1993).

Collectins identify carbohydrates present on microbial surfaces and bind via the CRD in a calcium-ion-dependent manner (Dec *et al.*, 2012). These interactions can induce agglutination or microbial neutralisation to prevent microbial spread. The interaction of collectin via CRD to the pathogenic microorganism, attract immune cells and this opsonisation may help in the phagocytosis of pathogenic microorganisms (Holmskov, 2000).



**Figure. 1.6. Structure of the collectins.** Collectins are composed of CRD region attached with  $\alpha$ -helical neck, a collagen-like domain and an N-terminal cysteins rich domain. Three neck regions combines to form a triple coiled shape and collagen regions assembles in to a triple helix. Cystine residues in the N-terminal region bound the subunits to form oligomeric structure (van *et al.*, 2004).

Surfactant proteins A and D and mannose binding lectins demonstrated a vital role as a fungal and other pathogens opsonins helping in the identification of the pathogen to control infection (Willment and Brown, 2008). Collectins stimulate complement fixation, ingestion of pathogens and cytokines production to control fungal infection (Brummer and Stevens, 2010).

### 1.16.1 Receptor for collectins on immune cells

Collectins are important molecules play an important role in innate immunity. They bind to the carbohydrates (antigen) on the surface of microbes, inducing aggregation and mediating phagocytosis through specific receptors on phagocytes (Holmskov, 2000). Collectins are involved in receptor-mediated phagocytosis of various microorganisms (Holmskov, 2000). There are various receptors that interact with collectins and are found on or are associated with the membranes of different cells responsible for stimulating phagocytes and promoting the phagocytosis, killing and clearance of microorganisms. SPA and SPD localization in endocytic vesicles and in lysosome granules of alveolar macrophages suggest that a receptor-mediated uptake of these two collectins occurs in these cells. Conglutinin has been found in the same

compartments of phagocytes and it has been speculated that this could result from an interaction mediated either by a receptors for conglutinin or indirectly via an iC3b receptor (Holmskov, 2000).

The collectin receptor (C1qR or C1q receptor or CD93) is express by many immune cells such as neutrophils, monocytes and lymphocytes (Tenner, 1993). It is known that this receptor binds with the collagen part of C1q and was originally purified from Raji cells (Ghebrehiwet *et al.*, 1984). Due to the binding of this receptor to MBL, SPA, conglutinin and CL-43, it is known as collectin receptor (Malhotra *et al.*, 1990). The C1q receptor was also shown to bind MBL, SPA, conglutinin and CL-43. Conglutinin have capability of binding with N-acetyl glucosamine, mannose, fucose residues and zymosan (Holmskov, 2000).

It has been found that cC1qR binds with in the N-terminal 54 amino acids of the bovine conglutinin. C1qR does not bind with the bovine conglutinin lacking of these residues (Malhotra *et al.*, 1993). C1q receptors stimulates different cellular immune responses results in the increase of phagocytic activity by macrophages, enhancement of fibroblast adherence, developing of oxidative burst in neutrophils, and stimulating of ingestion by endothelial cells. The same cellular response can be produced in results after the collectins binds with immune cells. It has been reported that C1q and MBL can act as opsonins that induce phagocytosis of apoptotic host cells infected by *E. histolytica* (Teixeira *et al.*, 2008).

Calreticulin (CRT) is a 60-kDa protein found on leukocytes, platelets and endothelium cells. It has been reported that this receptor interacts with collagen region of C1q. It has been found that CRT binds with other member of collectin family such as MBL, SP- A and bovine (Malhotra *et al.*, 1990). Glycoprotein-340 (gp-340) has been identified as a receptor for SPD, binds in the presence of calcium (Holmskov *et al.*, 1997).

### **1.16.2 Surfactant protein A and D**

SP-A and SP-D are pulmonary surfactants made of lipids (90%) and proteins (5-10%) produced by alveolar type II cells and non-ciliated bronchial epithelial cells (Clara cells) that cover the peripheral airways (Sano and Kuroki, 2004; Kishore *et al.*, 2006).

Both are hydrophilic surfactant proteins, with SP-A being the most abundant glycoprotein in surfactant. The lung collectins regulate phagocytosis and have the capacity of inducing agglutination of microorganisms via opsonisation so that this facilitates microbial recognition by alveolar macrophages and neutrophils (Sano and Kuroki, 2005).

SP-B and SP-C have shown a mechanistic role in modulating the surface tension of the pulmonary surfactant. SP-B and SP-C are small hydrophobic molecules whereas SP-A and SP-D are large hydrophilic in nature (Weaver and Whistsett, 1991; Kishore *et al.*, 2006). Both proteins alongside immune cells such as neutrophils and macrophages act as a first line of defence in the innate immunity. These proteins form a complex with lipids and proteins in the lungs, which reduces surface tension of alveoli and promote lung expansions (Espitia *et al.*, 2012). SP-A and SP-D identify particular carbohydrates present on microbes, which help in the elimination of the microbes from the host by opsonisation or agglutination mechanisms (Kishore *et al.*, 2005). These both have been shown to modulate the cytokines response such as enhancement of TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IFN- $\gamma$  production by human PBMCs (Kishore *et al.*, 2005).

SP-A is a 630-kDa protein, composed of six subunits whereas SP-D formed by four subunits and is a 520-kDa protein (Sano and Kuroki, 2005). SP-A and SP-D have similar overall structure composed of N-terminus, a triple-helical collagen region, an  $\alpha$ -helical coiled neck region and a globular structure at the C-terminus composed of carbohydrate which acts as ligands to pathogens (Kishore *et al.*, 2006). These proteins bind to pathogens and mediate the uptake of microbes by phagocytes (Espitia *et al.*, 2012).

The CRD region of the SP-A and SP-D is crucial for binding to pathogens and results in their clearance (Kishore *et al.*, 2006). Both SP-A and SP-D act as opsonins to present the pathogens to the dendritic cells and macrophages (Kishore *et al.*, 2006).

It has been known that SP-A binds with *M. bovis* BCG via CRD region and enhances the phagocytosis (Weikert *et al.*, 1997). SP-D can also bind to *M. tuberculosis* and can inhibit its uptake by macrophages (Ferguson *et al.*, 1999).

### 1.16.3 Conglutinin

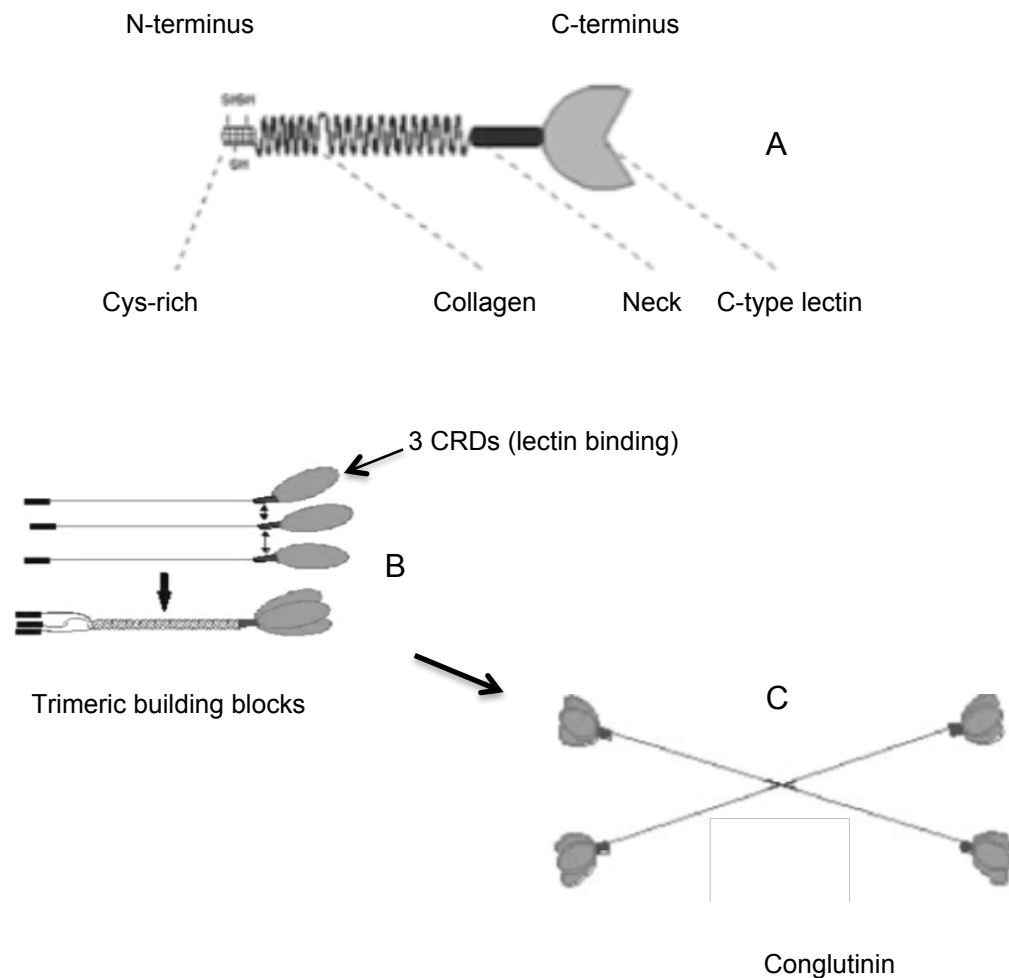
Ehrlich and Sach described conglutinin in 1902 first time as a heat-stable protein found in the bovine serum. In 1909 Bordet and Streng named a component found in the bovine serum which initiate the agglutination of erythrocytes coated with antibody and complement and called it conglutinin (Ingram and Barnum., 1965). Conglutinin is described as a member of collectin family of high molecular weight (Holmskov, 2000). Conglutinin also reported in mammals, chickens (Hogenkamp *et al.*, 2006), fish (Nakao *et al.*, 2006) and invertebrates (Nair *et al.*, 2000). Conglutinin is the member of C-type ( $\text{Ca}^{++}$  dependent) group of collectin family and is produced in liver (Holmskov, 2000). Collectin are playing an important role in innate immunity, they attached to carbohydrates of the microbial cell surface to induce aggregation in the presence of calcium to prevent the microbes growth (Holmskov, 2000). Collectin acts as secretory pattern opsonins to destroy the pathogens by activating phagocytic cells (Van *et al.*, 2004).

Conglutinin is a collectin detected in ruminant serum that participates in the agglutination of complement-coated erythrocytes. It has been demonstrated that conglutinin inhibits microbial and parasitic activity by its characteristics to bind with the saccharides present on microbial and parasitic surfaces (Lachmann, 1962). In ruminants, conglutinin is present in serum, neutrophils, follicular dendritic cells, macrophages those are generated from liver, lungs and spleen, thymus, lymph nodes and tonsils, which indicates conglutinin's important role in the immune responses (Holmskov *et al.*, 1992).

#### 1.16.3.1 Structure of bovine conglutinin

The gene encoding conglutinin is present on bovidae chromosome 28 (Liou *et al.*, 1994). Collectins including bovine conglutinin are oligomeric in structure assemble from various monomers. Conglutinin is a protein that forms a trimer that consists of monomers. These monomers are inter-connected and stabilised by covalent disulphide bridges (Hoppe and Reid, 1994). In electron microscope conglutinin revealed as an X-shaped tetramer, build of four basic structural units (Figure 1.7). This tetramer structure of conglutinin consists of four globular heads, attached to central hub by four collagen arms (Andersen *et al.*, 1992). Each monomer chain consists of 351

amino acid residues of N-terminal cysteine region, contains 25 residues followed by a 171-residue long collagenous region and a short 28-residue  $\alpha$ -helical neck region which is attached to the C-terminal (Lee *et al.*, 1991). SP-D and conglutinin show 78% residues resemblance (Lee *et al.*, 1991).



**Figure.1.7. Structure of bovine conglutinin: Conglutinin is an oligomer of polypeptide.** (A): conglutinin polypeptide consists of collagenous, neck and C-type lectin domain (CRD). (B): Three polypeptide chain of conglutinin combined to build a single subunit comprised of collagenous region and head comprised of three CRDs. (C): The subunits joined to form full size native bovine conglutinin (Hickling *et al.*, 2004).

### 1.16.3.2 Role of conglutinin in innate immunity

Lachman and Muller showed the conglutinin interaction with erythrocytes those are reacted with antibodies and complement was dependent on a factor in a serum, which

they named KAF (serine protease) (Lachman and Muller, 1968). In the presence of co-factors, it cleaves the  $\alpha$ -chain of C3b at two sites forms inactivated form of C3 known as iC3b (Laursen *et al.*, 1994).

It has been studied that this important molecule is synthesised by liver and secreted into the circulation where a wide range of its present in plasma, in female 1.25-35  $\mu\text{g/ml}$ , and in male 1.25-47  $\mu\text{g/ml}$  and mean concentration is 12  $\mu\text{g/ml}$  (Holmskov *et al.*, 1998). It has been seen that congenitinin levels in the serum of cows mainly effected by season, breeding and phases of reproductive cycle (Dec and Wernicki, 2006). Several studies reported that in abortion, calving and infection there was a decreased level of congenitinin in the serum. It has been reported that in a cow decrease in serum congenitinin started 4 week before calving and continue up to 2 weeks after delivery (Laursen *et al.*, 1994). The level started rising after delivery and reached up to its normal concentration in week 21 week after delivery (Ingram and Mitchell, 1970).

Congenitinin shows antibacterial activity against Gram negative bacteria and antiviral effect against influenza a virus and human immunodeficiency virus (HIV) (Laursen *et al.*, 1994).

Its function of agglutinating complement-coated erythrocytes is dependent on the presence of calcium and serum congenitinin activity factor (CAF), also termed as the factor I (Sliwa *et al.*, 2010) that plays an important role in the splitting of C3b component of the complement into inactive iC3b molecules, that further opsonises erythrocytes. However, incubation of antibody-coated erythrocytes for a long period of time with serum, by coated with congenitinin results in the splitting of iC3b to C3d and C3c, what in turn interferes with the agglutination properties of congenitinin that has demonstrated to bind to iC3b in a calcium-dependent manner (Sliwa *et al.*, 2010). It has been demonstrated that congenitinin binds with zymosan and complement component iC3b and has shown antimicrobial activity in vivo and vitro (Dec and Wernicki, 2006).

Bovine congenitinin has also been reported to inhibit IAV hemagglutination and infection activities by causing aggregation of viral particles, and act as an opsonin that



enhances neutrophil respiratory bursts responses after exposure to IAV (Hartshorn *et al.*, 1993).

### **1.16.3.3 Role of conglutinin in haemagglutination inhibition and Virus neutralization**

Bovine conglutinin binds with viral glycoprotein through their lectin domain and demonstrated haemagglutination inhibition activity. It has been reported that conglutinin shows haemagglutination inhibition and neutralization against the Nebraska calf diarrhea virus (NCDV). This activity proposed conglutinin role against rotavirus infections in cows (Reading *et al.*, 1998). Antiviral activities of conglutinin against influenza virus are well described although this virus is not involved in the infection of cattles. Conglutinin acts as  $\beta$ -inhibitors of influenza virus, which binds to viral carbohydrates and stimulate haemagglutination and neutralization. Conglutinin plays a significant role in neutralizing the virus, preventing viral growth and control the viral spreading to surrounding cells against influenza-A virus (Kawai *et al.*, 2007).

It has been studied that conglutinin binds to glycoprotein gp 160 present in the outer envelope of HIV-1 virus. The binding of bovine conglutinin to HIV-1 virus prevents binding with cell and stops infection (Andersen *et al.*, 1991).

### **1.16.3.4 Inhibition of bacterial growth by bovine conglutinin**

Conglutinin has been shown to increase mice survival from *Salmonella tyohimurium* when subcutaneously injected with conglutinin, with generation of reactive oxygen species (ROS) by granulocytes being one of the mechanisms by which conglutinin performs its antibacterial activity (Dec *et al.*, 2012).

It has also shown the protection against pathogen strains of *Pasteurella septica*, *Klebsiella pneumoniae*, *Listeria monocytogenes* and *Streptococcus pyogenes* in the mice (Ingram, 1959). Conglutinin also exhibit the anti-bacterial behavior against *Escherichia coli* and *Salmonella typhimurium* in vitro (Friis *et al.*, 1990).

The role of bovine conglutinin in bovine tuberculosis remains poorly understood. To characterise and understand the full role of conglutinin is key in order to understand the role that is plays in bovine tuberculosis.

### 1.17 Aims of study

In this study, we have to investigate the effect of recombinant conglutinin on mycobacterial growth. Bovine conglutinin is the first animal collectin to be discovered, it is structurally similar to human surfactant Protein D. SPD is known to interact with *Mycobacterium tuberculosis*, which is closely related to *Mycobacterium Bovis*, causative agent of bovine tuberculosis. Conglutinin is synthesized in the liver and is present in the serum. It is hypothesized that during inflammation conglutinin leaks from the blood vessels at the site of infection due to changes in vascular permeability and is likely to come in direct contact with *M. bovis* and immune cells and therefore may modulate the outcome of the infection. Its biological role is not fully understood. It is therefore interesting to study conglutinin's role in the bovine infection and immunity. The effects of these interactions were investigated using *M. smegmatis* and *M. bovis* BCG as model organism for *M. bovis*. The main aims of the study are:

**Aim 1:** Expression, purification and characterisation of the rfBC (conglutinin neck-CRD).

**Aim 2:** To investigate the direct effect of rfBC on the growth of *M. smegmatis* and *M. bovis* BCG.

**Aim 3:** To investigate the effect of rfBC on the uptake of *M. smegmatis* and *M. bovis* BCG by the THP-1 cells.

**Aim 4:** To investigate the effect of rfBC on the growth of *M. smegmatis* and *M. bovis* BCG by the THP-1 cells.

**Aim 5:** To investigate the effect of rfBC on the uptake of complement deposited *M. bovis* BCG by the THP-1 cells.

## **Chapter 2: Materials and Methods**

## 2.1 Bacterial cell culture media

### 2.1.1 Luria broth (LB) media

Liquid Luria broth (LB) media was prepared with 10g sodium chloride (NaCl), 10g tryptone and 5g-yeast extract dissolved in 1 litre distilled water. For solid agar media, 15g/L of agar were added, prior to autoclaving.

### 2.1.2 Mycobacteria growth

Middlebrooks 7H9 media (Sigma-Aldrich) was prepared by adding 0.2% (v/v) glycerol, 0.05% (v/v) Tween-80, and 10% (v/v) albumin-dextrose-catalase (ADC) (BD BBL, Becton Dickinson). *M. bovis* BCG (Pasteur strain) were grown by inoculating in 50ml Middlebrook 7H9 media contained all above supplements. GFP-expressing *M. bovis* BCG (Danish Strain 1331), containing the pGFPHYG2 plasmid (gift from Dr. B. Robertson, Imperial College, London), was grow in the above media but with the addition of 50 µg/ml of hygromycin to maintain the plasmid. Both were incubated in a shaking incubator (200 rpm) at 37<sup>0</sup>C for approximate 20 days. *M. smegmatis* (gift from Dr. Ansar Pathan, Brunel University) were cultured in 30ml LB media have 0.0016% (v/v) glycerol, 0.001% (v/v) Tween 80 and 100 µg/ml carbencillin (Sigma-Aldrich) and was incubated shaking incubator at 200rpm, at 37<sup>0</sup>C for 3-4 days till reach to exponential growth phase.

Middlebrook 7H10 agar base media (Sigma-Aldrich), supplemented with 0.2% (v/v) glycerol and 10% (v/v) OADC (Sigma-Aldrich) is used for bacterial growth on solid media.

## 2.2 Frozen storage of mycobacteria

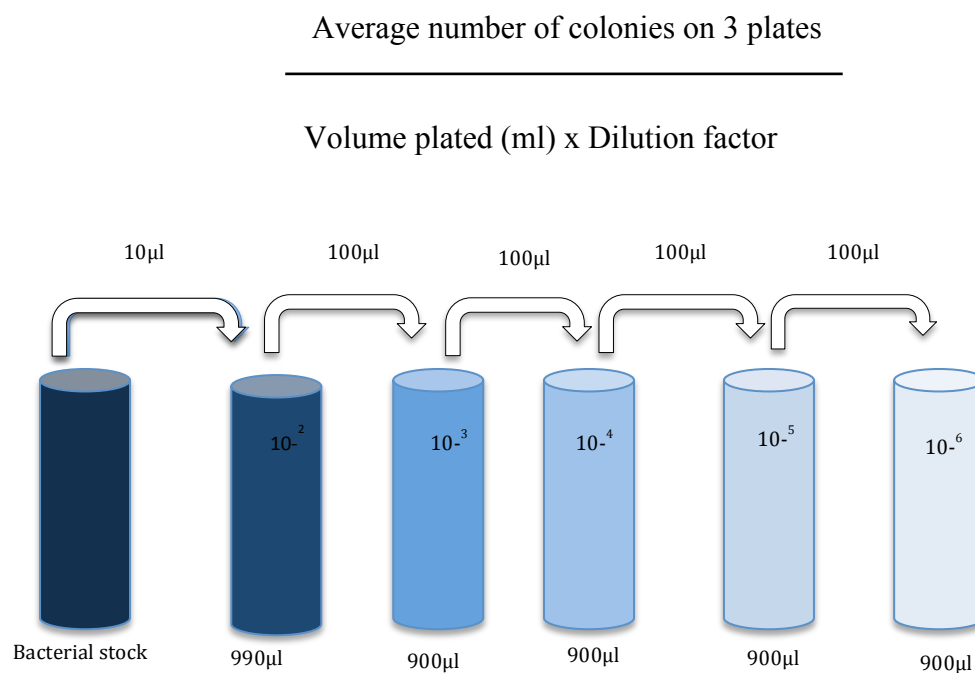
Mycobacterial cultures were grown and the optical density (OD) measured, OD<sub>600nm</sub>=0.6 to 1.00 to indicate the exponential phase of growth. For storage 30ml of culture of *M. bovis* BCG and *M. smegmatis* were centrifuged at 3000 rpm for 30 minutes. The pellet was mixed by gentle vortexing and then frozen with an equal volume of 100% sterile glycerol. Vials were then stored at -80<sup>0</sup>C.

### 2.3 Acid-Fast staining

Acid fast staining was performed to confirm *M. bovis* BCG and *M. smegmatis* and also to establish the purity of the culture before storage or its use in experiments. Acid fast staining was performed by using 500µl of mycobacterial culture ( $OD_{600nm} = 0.6-1.0$ ), which was spun down at 7000rpm for 10 minutes. The pellet was mixed and added on the slide. After drying the slide at room temperature, mycobacteria were fixed by passing the slide a few times through the flame until it dried. Carbol Fuschin stain (PRO-LAB), was applied on the smear covered by filter paper while heated under the flame for 5 minutes to enhance dye penetration. After 5 minutes of heating process the slide was washed through once with tap water followed by decolorizing solution (PRO-LAB). The bacterial smear was then flooded with methylene blue (PRO-LAB) and counter stained for 40 seconds. Any bacterial contamination in the mycobacterial suspension will be stained blue. Slide was then washed with tap water and left to air dry. Slides were then viewed using microscopy.

### 2.4 Colony Forming Units (CFU) counts of mycobacteria

CFU counts were performed to estimate the number of bacteria stored in vials at  $-80^{\circ}\text{C}$  for future experiments. One bacterial vial of each *M. smegmatis* and *M. bovis* BCG were, thawed on ice and sonication performed for 20 seconds using a CAMLAB, Transsonic T460 sonicator. We then added 10µl of sonicated sample into 990ml of LB for *M. smegmatis* or 7H9 for *M. bovis* BCG and diluted suspension to make a  $1 \times 10^{-2}$  dilution. A  $1 \times 10^{-3}$  dilution was then made by adding 100µl of bacterial suspension in to the 900 µl of plain media. The serial dilutions were carried out using the same method by transferring 100µl to add in to 900µl media until  $1 \times 10^{-6}$  serial dilutions was reached. For bacterial counting, 250µl from dilutions  $1 \times 10^{-3}$ ,  $1 \times 10^{-4}$ ,  $1 \times 10^{-5}$  and  $1 \times 10^{-6}$  were plated on an LB agar plates for *M. smegmatis* and 7H10 media plates containing 10% ADC for *M. bovis* BCG. *M. smegmatis* plates were incubated at  $37^{\circ}\text{C}$  for 3-4 days whilst *M. bovis* BCG plates were incubated for three weeks. Bacterial colonies were then counted by visual inspection. The following equation was used to count the bacteria in the original vial:



**Figure 2.1: Preparation of 10-fold serial dilutions of bacteria.**

## 2.5 Thawing and culturing of THP-1 cells

THP-1 cells (ATCC Catalog number TIB-202) were taken out from the liquid nitrogen containers and added into 10 ml of plain RPMI-1640 media (Gibco). The cells were centrifuged at 1500rpm for 10 minutes twice. THP-1 cells were cultured by adding 10ml of complete RPMI-1640 (cRPMI) (Gibco) consisting of 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100µg/ml penicillin (Sigma-Aldrich), 100µg/ml streptomycin (Sigma-Aldrich) and 1mM sodium pyruvate (Sigma-Aldrich) onto the cell pellet. After mixing of the cell pellet by vortexing, the cell suspension was transferred into a 25ml culture flask. These cells were kept in the CO<sub>2</sub> incubator at 37°C for 3 days to grow.

## 2.6 Transformation of competent cells

### 2.6.1 Preparation of competent cells

The *Escherichia coli* BL21 (DE3) pLysS strain (NEB) were used for transformation. A single colony from strain was picked from LB agar cultures and inoculated into 5 ml of LB media overnight. Then, 500µl of the primary culture was inoculated into 10 ml of fresh LB media and the cells grown with shaking at 37°C to an absorbance of between

0.3-0.4  $_{600\text{nm}}$ . The culture was then centrifuged at 3000 rpm for 10 minutes and 1ml of ice-cold 0.1 M calcium chloride added to the pellet, mixed gently, and the volume made up to 12 ml with 0.1 M calcium chloride. The sample was left in ice for an hour and then centrifuged at 3000 rpm for 10min and the supernatant discarded. Pellets were again resuspended in 1 ml of ice-cold 0.1 M calcium chloride. These cells were stored on ice in a cold room.

### 2.6.2 Transformation of competent cells

An aliquot of 200  $\mu\text{l}$  of competent cells was placed on ice and 2 $\mu\text{l}$  of the plasmid containing the conglutinin construct recombinant BK-neck-CRD in the pET vector (Wang *et al.*, 1995) (gift from Dr Uday Kishore) was added into the competent cells. Samples were placed on ice for 1 hour. After an hour of incubation on ice, the cells were incubated for 2 minutes at 42°C in water bath and then placed on ice for 5 minutes. 800 $\mu\text{l}$  of media was added into the sample and incubated the cell culture at 37°C for 45 minutes in a shaking incubator. Cells were then plated out on LB agar media consisting of 100 $\mu\text{g/ml}$  ampicillin and 50  $\mu\text{g/ml}$  chloramphenicol because the BL21 (DE3) pLysS strain of *E. coli* resistant to chloramphenicol and the conglutinin plasmid contains the ampicillin the resistance selective marker. Culture plates were incubated overnight at 37°C. The following day, colony growth was analysed.

### 2.6.3 Isolation of plasmid DNA

In order to maintain the conglutinin fragment plasmid, a single colony of *E. coli* TOP10 contains conglutinin fragment was inoculated from agar plates and in 5 ml of LB (containing 100  $\mu\text{g/ml}$  ampicillin) and the cells grown on a shaking incubator overnight at 37°C. Harvested 5ml of overnight bacterial culture by centrifugation. Resuspend the cell pellet in 250  $\mu\text{l}$  of Lysis buffer. The mixture was thoroughly mixed by inverting capped tube 6 times until the solution becomes viscous. Avoid doing vortex as this may result in shearing of the genomic DNA. Incubated the samples at room temperature for maximum 5 minutes. Then 250  $\mu\text{L}$  of precipitation buffer been added to the samples and mixed the solution until cloudy white precipitate is formed. Centrifuge the solution for 10 minutes at maximum speed to form pellet. Supernatant was transferred to miniprep column, stored in collection tube. Centrifuged the solution at 13000 rpm for 60 seconds. Flow-through was discarded from the collection tube and

column been inserted in same tube. Centrifuged the column at 13000rpm for 60 seconds after adding 750  $\mu$ l of wash buffer 1. Flow-through been discarded from the collection tube and 250  $\mu$ l of wash buffer 2 added in to the column. The samples were centrifuged at 13000 rpm for 60 seconds. Removed the column from the tube and the columns were then placed in fresh collection tubes and the DNA was eluted by adding 50 $\mu$ l elution solution to each column and spinning them at 13000 rpm for 60 seconds. Eluted plasmid DNA was stored at 4°C for immediate use and at -20°C for long-term storage.

## 2.7 Protein expression

In order to check the successful transformation of the conglutinin plasmid in the BL21 (DE3) pLysS strain of *E. coli*, pilot expression was performed. After confirming successful protein expression, large scale expression was then performed to purify the rfBC and maltose-binding protein (MBP).

### 2.7.1 Pilot expression of recombinant conglutinin (BK-neck-CRD) and MBP (Maltose binding protein).

Colonies from transformed BL21 (DE3) pLysS strain of *E. coli* cell culture containing the rfBC and maltose-binding protein (MBP) on agar plates were cultured in 5 ml LB (containing 100  $\mu$ g/ml ampicillin and 50  $\mu$ g/ml chloramphenicol). The cultures were then shaken overnight at 37°C and the following morning 500 $\mu$ l of each culture was grow in fresh 10 ml of LB containing 100  $\mu$ g/ml ampicillin and 50  $\mu$ g/ml chloramphenicol. The cells were grown on a shaking incubator at 37°C to an optical density (OD) of 600 nm of between 0.6 and 0.8.

0.4 mM of Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) (Sigma-Aldrich) was then added to the cells to induce protein expression. Additionally, 1 ml of culture, containing no IPTG was aliquoted and kept as an uninduced sample. Both induced and uninduced samples were shaken at 37°C for 3 hours. 1 ml from each induced sample was aliquoted into fresh microfuge tubes and together with the uninduced samples were centrifuged at 13000 rpm for 10 min. Pellets were then resuspended in 100  $\mu$ l of 2x treatment loading buffer (Table 2.1). The samples were then denatured at 100°C on a hot plate for 10 minutes and centrifuged for one minute at 13000 rpm.



Component	Concentration
1MTris-HCL (pH 6.8)	1.25ml
SDS (sodium dodecyl sulphate)	0.5g
2-ME (Mercaptoethanol)	1ml
Glycerol 100%	2ml
0.1% Bromophenol blue	0.4ml
Adjust the final volume to 10ml with ddH <sub>2</sub> O	

**Table 2.1: Composition of SDS-PAGE loading sample buffer (2X Treatment buffer)**

### 2.7.2 Expression and purification of the recombinant conglutinin (rfBC) at large scale.

BL21 (DE3) pLysS strain of *E. coli* (NEB) containing rfBC was cultured into 25ml of LB media added with 100 µg/ml ampicillin and 50 µg/ml chloramphenicol. The culture was shaken at 37°C, overnight. Then, 25ml of primary culture was added into 1 litre of LB with 100 µg/ml ampicillin and 50 µg/ml chloramphenicol. Bacterial cells were then grown at 37°C, while shaking to an OD 0.6-0.8<sub>600nm</sub>. Prior to the addition of IPTG to the culture, an uninduced sample was removed as a negative control for expression. To induce protein expression, 0.4mM of IPTG was added into the culture and incubated for a further 3 hours at 37°C in the incubator shaker at 200 rpm. The culture was harvested by centrifugation at 5000 rpm for 10 minutes. The cell pellet was then incubated with 50 ml of ice-cold lysis buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, containing 0.25% (v/v) Tween 20, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma) and 200µg/ml of lysozyme (Sigma) for 1 hour. The cell suspension was then sonicated at 60 Hz for 2 minutes with an interval of 1 minute on ice? (repeated 15 times). The sonicated sample was then spun down at 16,000 rpm for 30 minutes. The sonicated samples from the cell pellet were then analysed on a 12% SDS-PAGE to confirm that the recombinant conglutinin is present in the sample pellets. Samples expressing conglutinin were then subjected to purification. The protein samples were denatured and refolded by treating the bacterial suspension with 6 M urea buffer and reducing the concentration of urea. The starting buffer contained 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% (v/v) glycerol and 6 M

urea, which was used to mix the cell pellet and then dialysis performed against 1 litre of the same buffer but containing decreasing concentrations of urea to 4 M, 2M and 1 M urea. The final solution contained the soluble recombinant conglutinin protein, which was dialysed against ion-exchange starting buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl<sub>2</sub>, pH 8). Additionally, this solution was loaded onto the maltose agarose column (20 ml) (Sigma) for binding of rfBC with column. The column used was pre-washed with 50 ml of autoclaved water, 50 ml of salt buffer (50mM Tris-HCL, 1 M NaCl, 5mM CaCl<sub>2</sub>, pH 8) and 50 ml of affinity buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl<sub>2</sub>, pH 8).

After loading the protein sample, the column was treated with 30 ml of salt buffer and 15 ml of affinity buffer. The proteins were eluted with the elution buffer containing 50 mM Tris-HCL, 100 mM NaCl, 5mM EDTA, pH 8, NaN<sub>3</sub> 0.05%. The eluted conglutinin was collected into the 1.5ml fractions in microfuge tubes and analysed on a 12% (w/v) SDS-PAGE to confirm the presence of conglutinin. The OD<sub>280</sub> was determined for the concentration of the proteins before running the samples on SDS-PAGE to confirm the protein. Recombinant conglutinin was characterised using Western blotting and ELISA and identified by rabbit anti-bovine conglutinin IgG (gift from Dr. Uday Kishore).

### **2.7.3 Large scale expression and purification of maltose-binding protein (MBP)**

A single colony from of maltose binding protein (MBP) was inoculated into 25 ml of LB containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol and was shaken at 37°C overnight. The next day 25 ml of this culture was added into 1 litre LB (containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol) and grown in a 37°C shaking incubator until the OD reached between 0.6 to 0.8. To induce protein expression, 0.4 mM of IPTG was added to the sample. After 3 hours of incubation at 37°C, shaking at 200 rpm speed, the cell pellet was collected by centrifugation at 5000 rpm for 10 minutes.

50 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EGTA pH 7.5, 1 mM EDTA pH 7.5, 5% v/v glycerol, 0.2% v/v Tween 20, 0.1 mM PMSF and lysozyme (100 µg/ml)) was added to the cell pellet 4°C for 1 hour, in order to achieved lysis of the bacterial cell. The cell suspension was sonicated at 60 Hz for 2 minutes

with an interval of 1 minute (15 cycles). This sonicated sample was centrifuged at 16,000 rpm for 30 mins. The cell supernatant was collected and diluted 5 times with 50 ml of buffer I (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA PH 7.5, 0.2% v/v Tween 20 and 5% v/v glycerol). The diluted supernatant was then added to an amylose resin column (New England Bio labs). After passing through the MBP protein sample, the column was washed with 150 ml of buffer I (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA PH 7.5, 0.2% v/v Tween 20 and 5% v/v glycerol) and then buffer II (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA PH 7.5, and 5% v/v glycerol). The MBP were eluted by elution buffer (100 mM maltose, 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA PH 7.5 and 5% v/v glycerol). The OD<sub>280</sub> was determined for the concentration of the proteins before running the samples on SDS-PAGE to confirm the protein.

#### **2.7.4 Removal of endotoxin from rfBC**

rfBC was further treated to make them free of lipopolysaccharides (LPS). Polymyxin B column (Sigma-Aldrich) was cleaned by passing through 50 ml of 1% sodium deoxycholate (Thermo scientific) and 50 ml of autoclaved water. rfBC was mixed with the polymyxin B column beads and kept at roller shaker for 1 hour. After an hour, proteins were eluted and concentration was checked by a Nano drop spectrophotometer.

### **2.8 Protein characterization**

#### **2.8.1 SDS-PAGE .**

The purified sample of rfBC was verified by running purified samples on a 12% SDS-PAGE in order to determine the correct molecular weight. The molecular weight of the protein was compared to a protein marker (Bio Rad).

<b>Acrylamide Percentage</b>	<b>12%</b>
H <sub>2</sub> O	1.6ml
Acrylamide/Bis-acrylamide	2ml
1.5M Tris (pH 8.8)	1.3ml
10% (w/v) SDS	0.5ml
10% (w/v) APS	0.5ml
Temed	0.002ml

**Table 2.2: Components of resolving gel for SDS-PAGE**

<b>Acrylamide Percentage</b>	<b>12%</b>
H <sub>2</sub> O	0.68ml
Acrylamide/Bis-acrylamide	0.17ml
1.5M Tris (pH 8.8)	0.13ml
10% (w/v) SDS	0.01ml
10% (w/v) APS	0.01ml
Temed	0.001ml

**Table 2.3: Components of stacking gel for SDS-PAGE**

Proteins were diluted in 1:1 v/v ratio in 2x Laemmli buffer [4% (w/v) SDS, 20% glycerol, 10% 2-mercaptoethanol (v/v), 0.004% bromophenol blue (w/v), 0.125 M Tris HCL pH 6.8 (w/v)]. Samples were heated for 10 minutes at 100°C on heating block in to denature proteins. rFBC were loaded onto the wells. The gel was run at 120 volts for 90 minutes. Following this, the gel was stained with staining solution (50% methanol, 10% v/v acetic acid, 40% water with 0.1% coomassie blue) overnight on a rocking shaker. The next day, the gel was washed with distilled water and destained in destain solution (50% methanol, 10% acetic acid and 40% dH<sub>2</sub>O) for 2 hours. The gel image was captured using a Molecular Imager (Bio Rad).

### 2.8.2 Western blot

A western blot was used to detect the presence of the conglutinin proteins using a specific antibody. Onto 12 % SDS-PAGE gel 5, 10 and 20 µg/ml of recombinant conglutinin were loaded, together with the same concentration of MBP protein (negative control). A prestained protein ladder (Page Ruler™) with a mass range of 10–250 kDa was used as a molecular weight standard.

Following SDS-PAGE, the gels were equilibrated in blotting buffer (12 mM Tris, 96 mM glycine, 20% methanol, pH 8.3) and then the proteins were electro-blotted (Mini Trans-Blot Cell apparatus, Bio-Rad) onto polyvinylidene difluoride (PVD) membranes. Once the transfer was completed the PVD membrane was removed. Membrane was prepared for immunostaining by blocking with low-fat 5% milk (w/v) in PBS, overnight.

The membrane was washed three times (5 minutes each time) with wash buffer (PBS/0.05% Tween 20) and then incubated for one hour at room temperature with rabbit anti-bovine conglutinin IgG antibody (diluted 1:1000 in PBS). The membrane was washed 3 times (5 minutes each) with the wash buffer and the membrane was incubated for an hour with Protein A? polyclonal goat anti-rabbit IgG (PA-HRP) (diluted 1:1000 in PBS). The membrane was washed a further 3 times with the wash buffer for 5 minutes each. Blots were developed using Clarity Western ECL (BioRad) according to the manufacturer's instructions.

### **2.8.3 Enzyme-linked immunosorbent assay (ELISA)**

A 96 well ELISA plate (Fisher Scientific) was coated with serial dilutions of 0.5, 0.25, 1.25 and 0.65  $\mu\text{g/ml}$  of rfBC in the presence of 5 mM calcium chloride and same concentrations of MBP (contro) incubated for two hours (one hour at 37<sup>0</sup>C and one hour at 4<sup>0</sup>C). After 2 hours of protein incubation, the plate was washed twice with 200 $\mu\text{l}$  per well with PBS-tween (PBS/0.05% Tween 20). The plate was blocked with 200 $\mu\text{l}$  per well of 1% w/v BSA in PBS for 2 hours at room temperature. After blocking the contents from plates were discarded and washed three times with PBS/Tween 20. 100 $\mu\text{l}$  of the primary antibody: rabbit anti-bovine conglutinin IgG (diluted 1:1000 in PBS) was added to each well and the plate was incubated for an hour at room temperature. After the incubation contents from the plate were discarded and plate was washed 3 times with PBS/Tween 20. Protein A (1:1000) dilution was bind with rabbit anti-bovine conglutinin IgG, was added and incubated the sample at 37<sup>0</sup>C for an hour then the wells emptied and washed for three times. Substrate TMB (3, 3', 5,5'-Tetramethylbenzidine) was prepared as recommended by manufacturer, and 100 $\mu\text{l}$  was added per well. The ELISA plate was incubated in dark for 1-5 minutes to allow colour to develop. As soon as colour developed, the reaction was stopped by adding 50 $\mu\text{l}$  of

H<sub>2</sub>SO<sub>4</sub>. The absorption was read at 450 nm using an ELISA reader (Bio-Rad micro plate reader).

#### 2.8.4 Cross linking

In order to see whether rFBC forms dimer and trimer to stabilise the conglutinin structure as in native form. In order to do this, prepared cross linker BS<sup>3</sup> (bis<sup>3</sup> sulfosuccinimidyl suberate) (Thermo fisher, cat no; 21580) immediately before use. To make working concentration 1mM BS<sup>3</sup> from 1g powders, first 25mM BS<sup>3</sup> stock been made by dissolving 2mg BS<sup>3</sup> in to 140µl of DMSO (Dimethyl sulfoxide) according to manufacturer guideline. To make the final concentration of 1mM, added 1µl from 25mM stock to 24µl of DMSO (Dimethyl sulfoxide).

Seven aliquots of proteins were made in 1.5ml micro centrifuge tubes by adding 45µl of recombinant conglutinin (0.52µg/ml) for 7-time points reaction, 0,1,2,4,8,16 and 32 minutes. Added 5µl of 1mM of BS<sup>3</sup> in all micro centrifuge tubes except 0 time point. Incubate the reaction mixture at room temperature for different time points. After incubation for each time point, 20mM Tris-HCl (pH 7.5) were added to stop the reaction. To visualize the crosslinking of recombinant bovine conglutinin, the samples were ran on 12% SDS-PAGE.

#### 2.9 Preparation of *M.smegmatis* and *M. bovis* BCG bacteria for Enzyme-linked immunosorbent assay (ELISA) for binding assays

*M. smegmatis* (gifted by Dr Ansar Pathan) was inoculated in a 50 ml tube containing 30 ml of LB, 50µl glycerol, 30 µl Tween 80 and 1.5ml carbenciline (Sigma-Aldrich C1613) to avoid the contamination of fungus or non-specific bacterial cells. The cells were grown on a shaking incubator at 37°C to an OD of between 0.8 and 0.9 to reach its exponential phase. It took an average of 72 hours to reach at required OD. The culture was then centrifuged at 5000 rpm for 30 min and the supernatant discarded and the collected pellet stored at -80°C in glycerol solution (glycerol and LB 1:1 (v/v)).

*M. bovis* BCG (Pasteur strain) were grown as described in the method section 2.1.2. The bacteria were grown until they reached exponential phase, an OD<sub>600nm</sub> = 0.60-1.00, which is equivalent to 1 x 10<sup>9</sup> bacteria/ml (Abdul-Aziz *et al.*, 2016). The bacteria were

spun down at 4500 rpm for 10 minutes and washed three times in 0.15 mM PBS. The mycobacterial pellet was re-suspended in the 7H9 growth medium (with 10% ADC, 0.05% (v/v) Tween 80 and 0.2% (v/v) glycerol).

### **2.9.1 rfBc binding with *M. bovis* BCG and *M. smegmatis* cells by ELISA**

Microtitre wells were coated overnight with  $10^7$  *M. Bovis* BCG cells (50µl per well) and  $10^7$  *M. smegmatis* cells in 0.1 M carbonate /bicarbonate buffer, pH 9.6 and left at 4<sup>0</sup>C. The wells were blocked for 2 hours at 37<sup>0</sup>C with BSA then the plate washed twice with 200 µl phosphate-buffered saline (PBS). Different concentrations of rfBC (40, 20, 10, 5, 2.5, 1.25 and 0.625 µg) were set up as serial doubling dilutions in calcium buffer (40mM Tris, 200mM NaCl, 5mM CaCl<sub>2</sub>), and EDTA buffer (40mM Tris, 200mM NaCl, 5mM EDTA). Identical serial dilutions of MBP protein (40, 20, 10, 5, 2.5, 1.25 and 0.625 µg) were used as a negative control protein to be used with bacterial cells. rfBC was incubated with *M. bovis* BCG and *M. smegmatis* for 1 hours at 37<sup>0</sup> C and 1 hour at 4<sup>0</sup>C, then washed the plate with 200µl of washing buffer (PBS Tween 0.05%) for four times. The primary antibody, rabbit anti-bovine conglutinin IgG, (diluted in to PBS 1:1000) (100 µl/well) was incubated with the bacterial sample at 37<sup>0</sup>C for 1 hour. Again, the sample was washed with 200 µl per well of washing buffer (PBS/0.05% Tween 80) three times. Protein A diluted into PBS (1:1000) was added and incubated the sample at 37<sup>0</sup>C for 1 hour then the wells emptied and washed for three times. Development of the assay was completed by adding 100µl of 3, 3', 5,5'-Tetramethylbenzidine or TMB substrate in to the well. The sample was kept in dark for 1-5 minutes to allow colour to develop. The reaction was stopped by adding 50µl of H<sub>2</sub>SO<sub>4</sub> after the colour development. The absorption was read at 450 nm using a micro plate reader reader (Bio-Rad).

### **2.10 rfBC binding with *M. bovis* BCG cells microscopy**

This method was used to examine the binding of recombinant bovine conglutinin to the *M. bovis* BCG cells.  $2 \times 10^9$  *M. bovis* BCG cells were harvested at exponential phase, at an OD<sub>600nm</sub> =1. Dissolved bacterial cell pellets in to the 500µl 5mM calcium chloride (CaCl<sub>2</sub>) buffers contains 20µg/ml rfBC and 20µg/ml of MBP. Proteins were incubated with *M. bovis* BCG cells for 1 hour at 37<sup>0</sup>C in a shaker and 1 hour at 4<sup>0</sup>C. After 2 hours incubation samples were centrifuged at 7000 rpm for 10 minutes. Washed the

samples twice with 500µl/ml PBS (each time centrifuged at 7000 rpm for 5 minutes). The primary anti-body Rabbit anti-bovine conglutinin IgG diluted in to PBS (1:200) was added to conglutinin bound *M. bovis* BCG and incubated for 1 hour at 37<sup>0</sup>C. The samples were washed 3 times (500µl/ml PBS, each time centrifuged at 7000 rpm for 5 minutes). Protein A conjugated to FITC (Sigma) (1:1000) was treated to the complex in both samples. The samples were incubated for 30 minutes in the dark at room temperature. The cells were harvested after washing 3 times with PBS. Cell suspension (20µl) was added on a clean glass slides and covered with a coverslip. Leica microscope (Leica DM4000) was used to visualised the slides.

### **2.11 Direct effect of rFBC to *M. smegmatis* and *M. bovis* BCG growth**

*M. bovis* BCG and *M. smegmatis* were grown in 7H9 and LB media respectively as describe above. At OD<sub>600nm</sub>=1.00, which is equivalent to 1x10<sup>9</sup> bacteria/ml. Taken 1ml of *M. bovis* BCG and *M. smegmatis* in 2ml centrifuge tube and centrifuged at 10,000rpm for 10 minutes. Cell pellets were suspended into 1ml of LB media for *M. smegmatis* and 1ml of 7H9 for *M. bovis* BCG. To break the clumps in the cultures, passed through 12–15 times from 1ml syringe with 25 gauge needle. After vortexing the samples, 100µl of bacterial culture was diluted in 900µl of LB or 7H9. A bacterial dilution of 1x10<sup>-5</sup> and 1x10<sup>-3</sup> in total volume of 1ml of LB or 7H9 medium was used for *M. smegmatis* and *M. bovis* BCG respectively, in the experiment.

Three different concentrations of conglutinin: 10, 20 and 40µg/ml and one control sample without protein, were incubated with *M. smegmatis* and *M. bovis* BCG cells for 1 hour at 37<sup>0</sup>C and 1 hour at 4<sup>0</sup>C in the shaker. After 2 hours of incubation, 200µl bacterial culture was plated on each plate. LB agar plates were used for *M. smegmatis* and 7H10 plates (with 10% OADC) were used for *M. bovis* BCG cultures. *M. smegmatis* were kept in 37<sup>0</sup>C incubator for three days and *M. bovis* BCG plates were kept on same conditions, but for approximately 2-3 weeks for growth. After the appearance of bacterial colonies, they were counted and analysed.

### **2.12 Measuring *M. bovis* BCG cell lysis by propidium iodide uptake**

For this study, we took 1 ml of *M. bovis* BCG culture at OD<sub>600nm</sub>=1.0 and centrifuged at 7000 rpm for 10 mins. The *M. bovis* BCG pellet was resuspended in 200µl of 5 mM



CaCl<sub>2</sub>. Three different concentration (10, 20 and 40µg/ml) of rfBC added in three microfuge tubes and two were left untreated with protein. All samples were incubated 1 hour at 37°C in the shaker and one hour at 4°C. After two hours incubation, the sample was centrifuged at 7000 rpm for 10 minutes. Bacterial pellets were then suspended in to 1 ml of PBS. One of untreated sample of *M. bovis* BCG was heated on hot plate at 100°C for 10 minutes to lyse the cell membrane as a positive control for the assay.

Propidium iodide (Sigma-Aldrich) (1:1000) was added to the bacterial suspension. Cells were incubated with propidium iodide for 20 minutes at room temperature in the dark. After incubation, samples were washed with 500 µl of PBS three times by centrifugation at 7000 rpm for 10 minutes.

A small amount of the cell suspension (20µl) was added on a clean glass slide and covered with a coverslip. Slides were visualised using a fluorescence microscope (Leica DM4000).

## 2.13 Infection Assays

### 2.13.1 Infection Assay by Colony forming Units (CFU)

THP-1 macrophage cells were grown in complete RPMI media as described in method section 2.5. After the growth, cells were adjusted to  $7 \times 10^5$  cells/well and were differentiated into macrophages by adding 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and then incubated for 48 hours in 12-well plates. After 48 hours, cells were washed once with plain RPMI and media replaced with fresh complete RPMI (cRPMI) for overnight. Cells were then washed once with plain RPMI and then left in 400 µl of plain RPMI (serum free, antibiotic free) at least an hour before adding bacteria.

*M. bovis* BCG were grown to OD<sub>600</sub>=0.6-1.0 at log phase (stationary culture) as described above. 2 ml of culture was centrifuged with an OD<sub>600nm</sub> = 0.6 in 2 ml microfuge tube at 3300 x g for 10 minutes (The following formula was used for consistency, if culture has an OD<sub>600</sub> of 0.5, then need to spin down 2.4 ml ( $0.6 \times 2 \text{ ml} / 0.5 = 2.4 \text{ ml}$ ). After harvesting bacteria, the bacterial pellet was resuspended in 200

µl of plain RPMI with no supplements. To ensure a single-cell suspension, the bacteria were passed through from 1ml syringe with 25-gauge needle, 12–15 times.

Tubes were set up with 10, 20 and 40 µg/ml of conglutinin added to bacteria, together with 5mM of CaCl<sub>2</sub> (10µl of 0.1 M CaCl<sub>2</sub>). Samples were incubated on a shaking incubator at 37°C for 1 hour followed by 1 hour at 4°C with shaking to allow conglutinin to bind. After 2 hours incubation with conglutinin, bacterial cells were centrifuged at 3300 x g for 10 minutes and then gently resuspended in 2 ml of plain RPMI.

70µl of the bacterial suspension with and without conglutinin was added to the THP-1 cells giving a multiplicity of infection (MOI) of 10:1, (bacteria:cells). Bacteria were then mixed with THP-1 cells by gentle pipetting in the wells. Assays were then incubated at 37°C and 5% CO<sub>2</sub> for 3 hour, 6 hour, 24 hour and 48 hour time-points.

After each time-point of incubation, the supernatant was collected for cytokine protein analysis. THP-1 cells were then washed 3 times with plain RPMI and removed using 0.25% of trypsin and incubated at 37°C for 10 minutes. Then 500µl of RPMI with serum was added to neutralize the effect of trypsin. Cells were then centrifuged at 1400 rpm for 10 minutes and the supernatant discarded. At the 3- and 6-hour time-points, THP-1 cells were lysed and colony-forming unit assay performed to investigate the number of intracellular bacteria (see below). For the remaining time-points, supernatants and pellets were collected and stored at -80°C for further experiments.

For the 3- and 6-hours timepoints, cell pellets were harvested and resuspended in 1ml 0.1% saponin to lyse the THP-1 cells. The cells were vortex for 10 minutes. Ten-fold Serial dilutions were then made by adding 100µl of the suspension into 900µl of 7H9 to give a  $1 \times 10^{-1}$  dilution. Two further 10-fold dilution were carried out to give a  $1 \times 10^{-2}$  and  $1 \times 10^{-3}$  dilutions. 200µl was plated on 7H10 plates supplemented with 10% OADC at each dilution. Assays were conducted in triplicate. 7H10 plates were incubated for 2-3 weeks at 37°C and then bacterial colonies counted and analysed.

### 2.13.2 Infection assay by microscopy

THP-1 cells were grown in cRPMI as described in method section 2.5. After growth, THP-1 cells were re-suspended in cRPMI seeded at  $1 \times 10^5$  cells/well on sterile coverslips (Sigma-Aldrich) and were induced to adhere with by adding 100 ng/ml of PMA. Cells were incubated for 48 hours in 12-well plates. After 48 hours, cells were washed three times with plain RPMI and media replaced with 1 ml of cRPMI overnight. Cells were then washed with plain RPMI and replaced and left in 400  $\mu$ l of plain RPMI (serum-free, antibiotic-free) for 1 hour before the addition of bacteria.

*M. bovis* BCG-GFP (strain expressing GFP plasmid pGFPHYG2; gift from Dr B. Robertson, Imperial College London) was grown in Middlebrook 7H9 media (Sigma Aldrich) as described above. *M. bovis* BCG-GFP were grown to  $OD_{600}=0.6-1.0$  at log phase (stationary culture) as described above. 2 ml of culture was centrifuged with an  $OD_{600nm} = 0.6$  in 2 ml microfuge tube at  $3300 \times g$  for 10 minutes (The following formula was used for consistency, if culture has an  $OD_{600}$  of 0.5, then need to spin down 2.4 ml ( $0.6 \times 2 \text{ ml}/0.5 = 2.4 \text{ ml}$ ). After harvesting bacteria, the bacterial pellet was resuspended in 200  $\mu$ l of plain RPMI with no supplements. To ensure a single-cell suspension, the bacteria were passed through from 1ml syringe with 25-gauge needle, 12–15 times

Tubes were set up with 10, 20 and 40  $\mu$ g/ml of conglutinin added to bacteria, together with 5mM of  $CaCl_2$  (10 $\mu$ l of 0.1 M  $CaCl_2$ ). Samples were incubated on a shaking incubator at 37°C for 1 hour followed by 1 hour at 4°C with shaking to allow conglutinin to bind. After 2 hours incubation with conglutinin, bacterial cells were centrifuged at  $3300 \times g$  for 10 minutes and then gently resuspended in 2 ml of plain RPMI.

70 $\mu$ l of the bacterial suspension with and without conglutinin was added to the THP-1 cells giving a multiplicity of infection (MOI) of 10:1, (bacteria: cells). Bacteria were then mixed with THP-1 cells by gentle pipetting in the wells. Assays were then incubated at 37°C and 5%  $CO_2$  for 3 hour and 6 hour.

After 3 hours of incubation, the supernatants were discarded from the wells. Coverslips were then washed 3 times with 500  $\mu$ l PBS in wells. Coverslips were then fixed with

300  $\mu$ l of 4% paraformaldehyde (PFA) for 10 minutes at room temperature. After fixing, cells were then washed 3 times with 500  $\mu$ l of PBS.

A master mix containing PBS, Hoechst (1:10000) and wheat germ agglutinin (WGA) (Thermo Scientific) (1:500) was prepared. 50 $\mu$ l of the master mix was added to each coverslip and incubated for 10 minutes at room temperature in the dark. Coverslips were then washed 5 times with 1ml of PBS and placed on the glass microscope slides for visualisation using a fluorescent microscope (Leica DM4000 microscope). Few drops of antifade (Citifluor AF3) were added on each coverslips and slides covers were used on top of cover slips.

#### **2.14 Nuclear Factor Kappa B (NF- $\kappa$ B) Translocation Assay**

To examine the translocation of NF- $\kappa$ B, THP-1 cells were grown in cRPMI as described in method section 2.5. After growth, THP-1 cells were re-suspended in cRPMI seeded at  $1 \times 10^5$  cells/well on sterile coverslips (Sigma-Aldrich?) and were induced to adhere with by adding 100 ng/ml of PMA. Cells were incubated for 48 hours in 12-well plates. After 48 hours, cells were washed three times with plain RPMI and media replaced with 1 ml of cRPMI overnight. Cells were then washed with plain RPMI and replaced and left in 400  $\mu$ l of plain RPMI (serum-free, antibiotic-free) for 1 hour before the addition of bacteria.

1.2 ml of *M. bovis* BCG-GFP culture with OD600 = 1.0 was centrifuged in six different 2 ml microfuge tubes at 3300 g for 10 minutes. Six conditions were studied: 1: *M. bovis* BCG only, 2: *M. bovis* BCG+complement activated, 3: *M. bovis* BCG+conglutinin 20  $\mu$ g, 4: *M. bovis* BCG+40  $\mu$ g conglutinin, 5: *M. bovis* BCG+complement activated+20  $\mu$ g conglutinin and *M. bovis* BCG+complement activated+40  $\mu$ g conglutinin.

For complement activation (sample tubes 2, 5 and 6) bacterial pellets were dissolved in 1:5 serum dilution and 160 $\mu$ l DGVB<sup>++</sup>Mg EGTA buffer). They were incubated for 45 min at 37°C on a shaker to activate alternative complement pathway and deposited complement protein on *M. bovis* BCG. After the deposition of complement proteins on the *M. bovis* BCG surface, all the samples were spun at 3300xg for 10 minutes. Bacterial cell pellets were then resuspended in 200  $\mu$ l of 7H9 media containing 5mM

CaCl<sub>2</sub> 20 and 40 µg/ml of conglutinin were then added in to the bacterial suspensions of complement deposited bacteria (5 and 6 sample tubes respectively) and no complement deposited (3 and 4 sample tubes). All samples were incubated in a shaking incubator at 37°C for 1 hour followed by 1 hour at 4°C with shaking. After 2 hours samples were centrifuged at 3300 x g for 10 minutes. 2 ml of plain RPMI was added to the pellet and gently resuspended the bacteria into a single-cell suspension using a 1ml syringe with 25-gauge needle.

70µl of the bacterial suspension with and without rFBC was added to the THP-1 cells giving a multiplicity of infection (MOI) of 10:1, (bacteria:cells). Bacteria were then mixed with THP-1 cells by gentle pipetting in the wells. Assays were then incubated at 37°C and 5% CO<sub>2</sub> for 6 hour.

After 6 hours of treatment, cells were washed 3 times with 500 µl PBS inside the wells. Cover slips were fixed by adding 500 µl of 100% Methanol and incubated at -20°C for 10 minutes. The coverslips were washed three times after fixation of cells with 500 µl of PBS buffer. NF-κB primary antibodies (1:200) was added to each coverslip and left for 1 hour at RT. 500 µl of PBS was used to wash the coverslips, 3 times after primary antibody treatment.

A master mix containing Hoechst (1:10000) and Cy3 secondary antibody for NF-κB (1:500) in PBS was prepared. 50 µl of master mix of both was added to each coverslip and left for 1 hour at RT. Then the coverslips were washed four times with 1 ml of PBS. Slides were prepared to view under the Leica DM4000 Fluorescence microscope by adding antifade (Citifluor AF3) and placing covers on the top of slides.

## **2.15 Complement activation assay**

### **2.15.1 Serum adsorption**

Normal Human Serum (TCS Biosciences Ltd) was incubated with Sheep Blood in Alsever's Solution (Thermo scientific) to reduce Anti-Forssman antibody activity. Sheep Erythrocytes were washed with PBS and centrifuged at 3,000 rpm for 5 min until no spontaneous lysis of erythrocytes occurred. 2.6 ml of washed red blood cells (RBC) were centrifuged and suspended in 13 ml of normal human serum. The

suspension was incubated overnight at 4°C on a slow rotator. After overnight incubation, the serum was collected by centrifuged at 3000 rpm at 4°C for 5 minutes and serum supernatant was collected. Serum was tested for the presence of anti-Forsman antibodies by incubating 500 µl of erythrocytes (E cells) in 1:5 diluted sera (20 µl serum + 80 µl DGVB<sup>++</sup>) and 1:10 diluted sera (10 µl serum + 90µl DGVB<sup>++</sup>), and incubated for 1 hour at 37°C.

## 2.16 Haemolytic assay

### 2.16.1 Preparation of neuraminidase-treated sheep erythrocytes (NE)

For the haemolytic assay and preparation of neuraminidase-treated E cells, we followed same protocole as mentioned by Carroll, washed E cells were dissolved in incubation buffer containing 10mM sodium phosphate, 140 mM NaCl, 0.1 mM PMSF, pH 5.0 and the cells concentration standardised to  $1 \times 10^9$  cells/ml. For every  $2 \times 10^{10}$  cells, 5 units of neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) were added and incubated cells for 1 hour at 37°C in a shaking incubator . After 1 hour the cells were spun at 3000 rpm for 10 minutes and washed with PBS contains 0.5 mM EDTA. Cells were again washed with DGVB-Mg EGTA buffer. Neuraminidase-treated erythrocytes were finally dissolved into DGVB-Mg EGTA buffer and concentration adjusted to  $10^9$  cells/ml (Carroll *et al*, 2009).

### 2.16.2 Alternative pathway complement consumption assay

The experiment was performed with the serum sample kept at 4°C on ice until the haemolytic incubation. The experiments were performed in duplicates for each sample: 150 µl of diluted serum to suspended 100 µl, 200 µl, 400µl and 800µl of *M. bovis* BCG  $OD_{600nm} = 1$  and 150 µl of DGVB-MgEGTA (control) to monitor spontaneous haemolysis. They were incubated for one hour at 37°C on a shaker. After treatment, spun down (10,000 rpm, 5 min), 100 µl of the supernatant was added with 100µl of  $10^9$  cells/ml NE in DGVB-MgEGTA and the tubes were kept for 1 hour at 37° C. This stage of the haemolytic assay was performed to measure the total complement activity. After incubation, they were spun down at 2,500 rpm for 15 minutes. Absorbance of the supernatant was read at OD 541 nm.

### **2.17 Complement dependent interactions of *M bovis* BCG coated with rfBC with THP-1 cells and phagocytosis by CFU (Colony forming Units).**

THP-1 macrophage cells were grown in complete RPMI media as described in method section 2.5. The same numbers of THP-1 cells were grown, differentiated and seeded into the 12 well plates as mentioned in method section 2.13.1.

*M. bovis* BCG were grow to OD600=0.6-1.0 at log phase (stationary culture). This *M. bovis* BCG solution was separated into eppendorf tubes; 1: *M. bovis* BCG, 2: *M. bovis* BCG+Complemnt activated, 3: *M. bovis* BCG+Complemnt activated+conglutinin 10µg 4: *M. bovis* BCG=Complemnt activated+conglutinin 20µg and *M. bovis* BCG+Conglutinnin 40µg. Spun down 1.2 ml of culture with OD600 = 1.00 in 2 ml microfuge tube of all conditions at 3300 × RCF for 10 minutes. After harvesting cells, resuspended bacterial pelle + in to 200µl 1:5 serum dilution (40µl adsorbed serum and 160µl DGVB++MgEGTA buffer) except first condition without complement. They were incubated for 45 min at 37°C on a shaker to activate alternative complement pathway and deposited complement protein on *M. bovis* BCG. After incubation spun down samples at 3300xg for 10 minutes.

After harvesting bacteria, the bacterial pellet was resuspended in 200 µl of plain RPMI with no supplements. To ensure a single-cell suspension, the bacteria were passed through from 1ml syringe with 25-gauge needle, 12–15 times. Tubes were set up with 10, 20 and 40 µg/ml of conglutinin added to bacteria, together with 5mM of CaCl<sub>2</sub> (10µl of 0.1 M CaCl<sub>2</sub>). Samples were incubated on a shaking incubator at 37°C for 1 hour followed by 1 hour at 4°C with shaking to allow conglutinin to bind. After 2 hours incubation with rfBC, bacterial cells were centrifuged at 3300g for 10 minutes and then gently resuspended in 2 ml of plain RPMI.

70µl of the bacterial suspension with and without rfBC was added to the THP-1 cells giving a muliticity of infection (MOI) of 10:1, (bacteria:cells). Bacteria were then mixed with THP-1 cells by gentle pipetting in the wells. Assays were then incubated at 37°C and 5% CO<sub>2</sub> for 3 hour, 6 hour, 24 hour and 48 hour time-points.

After each time-point of incubation, the supernatant was collected for cytokine protein analysis. THP-1 cells were then washed 3 times with plain RPMI and removed using

0.25% of trypsin and incubated at 37°C for 10 minutes. Then 500µl of RPMI with serum was added to neutralize the effect of trypsin. Cells were then centrifuged at 1400 rpm for 10 minutes and the supernatant discarded. At the 3- and 6-hour time-points, THP-1 cells were lysed and colony-forming unit assay performed to investigate the number of intracellular bacteria (see below). For the remaining time-points, supernatants and pellets were collected and stored at -80°C for further experiments.

For the 3- and 6-hours timepoints, cell pellets were harvested and resuspended in 1ml 0.1% saponin to lyse the THP-1 cells. The cells were vortex for 10 minutes. Ten-fold Serial dilutions were then made by same way as mentioned in method section 2.13.1. 200µl was plated on 7H10 plates supplemented with 10% OADC at each dilution. Assays were conducted in triplicate. 7H10 plates were incubated for 2-3 weeks at 37°C and then bacterial colonies counted and analysed.

### **2.18 Complement dependent interactions of *M. bovis* BCG coated with rfBC with THP-1 cells and phagocytosis by microscope.**

1 x 10<sup>5</sup> cells/well of THP-1 were seeded, grown and differentiated on sterile coverslips same as mentioned in method section 2.13.2. *M. bovis* BCG GFP (strain producing GFP harbors plasmid pGFPHYG2) culture with OD600 = 0.8 is separated into five microfuge tubes; 1: *M. bovis* BCG, 2: *M. bovis* BCG+Complement activated, 3: *M. bovis* BCG+Complement activated+rfBC 10µg, 4: *M. bovis* BCG+Complement activated+rfBC 20µg and 5: *M. bovis* BCG+Conglutinin 40µg. All tubes were spun at 3300 × RCF for 10 minutes to collect the bacterial pellet. After harvesting cells, resuspended bacterial pellet in to 200µl 1:5 serum dilution (40µl adsorbed serum and 160µl DGVB++MgEGTA buffer) except first condition without complement. They were incubated for 45 min at 37°C on a shaker to activate alternative complement pathway. After incubation spun down samples at 3300xg for 10 minutes.

After harvesting bacteria, the bacterial pellet was resuspended in 200 µl of plain RPMI with no supplements. To ensure a single-cell suspension, the bacteria were passed through from 1ml syringe with 25-gauge needle, 12–15 times

Tubes were set up with 10, 20 and 40 µg/ml of rfBC added to bacteria, together with 5mM of CaCl<sub>2</sub> (10µl of 0.1 M CaCl<sub>2</sub>). Samples were incubated on a shaking incubator



at 37°C for 1 hour followed by 1 hour at 4°C with shaking to allow rfBC to bind. After 2 hours incubation with rfBC, bacterial cells were centrifuged at 3300x g for 10 minutes and then gently resuspended in 2 ml of plain RPMI.

70µl of the bacterial suspension with and without rfBC was added to the THP-1 cells giving a multiplicity of infection (MOI) of 10:1, (bacteria:cells). Bacteria were then mixed with THP-1 cells by gentle pipetting in the wells. Assays were then incubated at 37°C and 5% CO<sub>2</sub> for 3 hour and 6 hour.

After 3 hours of incubation, the supernatants were discarded from the wells. Coverslips were then washed 3 times with 500 µl PBS in wells. Coverslips were then fixed with 300 µl of 4% paraformaldehyde (PFA for 10 minutes at room temperature. After fixing, cells were then washed 3 times with 500 µl of PBS.

A master mix containing PBS, Hoechst (1:10000) and wheat germ agglutinin (WGA) (Thermo Scientific) (1:500) was prepared. 50µl of the master mix was added to each coverslip and incubated for 10 minutes at room temperature in the dark. Coverslips were then washed 5 times with 1ml of PBS and placed on the glass microscope slides for visualisation using a fluorescent micropose (Leica DM4000 microscope). Few drops of antifade (Citifluor AF3) were added on each coverslips and slides covers were used on top of cover slips.

## **2.19 Quantitative real-time polymerase chain reaction (q-PCR) to determine gene expression**

### **2.19.1 rfBC treatment of THP-1 cells with or without intracellular *M. bovis* BCG**

Inhibition assay were performed for intracellular *M. bovis* BCG according to the protocol mentioned in section 2.13.1 and 2.17. The experiment included THP-1 cells as a control, THP-1 cells with intracellular *M. bovis* BCG treated with 10µg/ml, 20µg/ml and 40µg/ml of rfBC. rfBC treatment times included 3 and 6 hours.

### 2.19.2 Designing Primers for target genes

Forward and Reverse Primers for various target genes were designed using Basic Local Alignment Search Tool (BLAST) and Primer-BLAST utility available at NCBI. (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences for the forward and reverse primers used for target genes are shown in Table 2.4. These primers were used in the previous studies in the lab (Kouser, L *et al*, 2018; Abdul-Aziz, M *et al*, 2016)

Target gene	Forward Primer	Reverse Primer
18S rRNA	ATGGCCGTTCTTAGTTGG TG	CGCTGAGCCAGTCAGTGTA
IL-1 $\beta$	GGACAAGCTGAGGAAGA TGC	TCGTTATCCCATGTGTGCGAA
TNF- $\alpha$	AGCCCATGTTGTAGCAA ACC	TGAGGTACAGGCCCTCTGAT
IL-10	TTACCTGGAGGAGGTGA TGC	GGCCTTGCTCTTGTTTTTCAC
IL-12	AACTTGCAGCTGAAGCC ATT	GACCTGAACGCAGAATGTCA
TGF- $\beta$	GTACCTGAACCCGTGTT GCT	GTATCGCCAGGAATTGTTGC
IL-6	GAAAGCAGCAAAGAGG CACT	TTTACCAGGCAAGTCTCCT

**Table 2.4: DNA Sequence for forward and reverse primers used for amplification of different target genes in THP-1 cells. All sequences are 5' to 3'.**

### 2.19.3 RNA extraction from THP-1 cells

Total RNA extraction from THP-1 cells was performed using the GenElute™ Mammalian Total RNA Purification Kit (Sigma-Aldrich) according to the manufacturer's protocol. THP-1 cells were first thawed on ice and 250  $\mu$ l of lysis solution containing 2.5  $\mu$ l 2-mercaptoethanol (2-ME) to the cell pellets. The mixture was thoroughly vortexed until all the clumps dissolved. To each cell lysate, 250  $\mu$ l of

70% ethanol was added and was vortexed briefly before loading onto RNA binding columns. The samples were centrifuged at 13000 rpm for 15 seconds. The column was washed once with 500µl of Wash Solution 1 at 13000 rpm for 15 seconds followed by washing twice with 500 µl Wash solution 2 for 15 seconds each. To remove any excess ethanol left from washing, the columns were spun for another 2 minutes at 13000 rpm. The columns were then placed in fresh collection tubes and the total RNA was eluted by adding 50µl elution solution to each column and spinning them at 13000 rpm for 1 minute. Eluted RNA was kept in a -80°C freezer until further use.

#### **2.19.4 DNase treatment**

The eluted RNA was made free of any contaminating DNA by using DNase I kit (Sigma-Aldrich). 50 µl of eluted RNA was treated with 5 µl of 10X buffer and 5µl of DNase I enzyme for 15 minutes at room temperature to allow digestion of any DNA present in the sample RNA. The reaction was stopped by adding 5µl of stop solution and transferring the samples to a heat block at 70°C for 10 minutes to inactivate the DNase I enzyme.

At this point the concentration eluted RNA was estimated by measuring the absorbance at 260 nm and the quality of eluted RNA was determined from the ratio of absorbance at 260:280nm using NanoDrop 2000/2000c (Thermo Fisher Scientific, UK).

#### **2.19.5 Synthesis of cDNA from total RNA**

High Capacity RNA to cDNA Kit (Applied Biosystems, USA) was used for the synthesis of cDNA from the purified eluted RNA by reverse transcription. A master mix consisting of 2X RT buffer (10 µl/sample) and 20X enzyme mix (1 µl/sample) was prepared. 11 µl of the master mix was added to each tube containing 9 µl of eluted RNA in PCR tubes on ice. Finally, the eluted RNA was converted into cDNA by placing the tubes in a thermal cycler (Peqlab). The samples were incubated at 37°C for 60 minutes and the reaction was terminated by heating the samples to 95°C for 5 minutes. The synthesized cDNA samples were stored at -20°C.

#### **2.19.6 Quantitative real time PCR (qPCR)**

The level of mRNA expression for various target genes were analysed using qPCR. The reactions were carried out in triplicate for each target gene in a MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems). For each well the reaction

mixture consisted of 5  $\mu$ l of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA), 0.15  $\mu$ l of 5  $\mu$ M gene specific forward and reverse primers respectively and 1  $\mu$ l (500ng) of relevant cDNA. The final concentration in each well was made 10 $\mu$ l by adding ultra-pure water (Sigma-Aldrich, UK). The plates were sealed using transparent adhesive sealing films and centrifuged at 1000rpm for 1 minute to settle the contents in the wells. Finally, the q-PCR plate was placed in a Step One Plus™ qPCR machine (Applied Biosystems). Initially the samples were incubated 2 min at 50°C followed by incubation at 95°C for 10 min. The template was amplified for 40 cycles under temperature conditions; 15s incubation at 95°C and 1 min at 60°C.

### 2.19.7 Data analysis

Human 18S rRNA gene was included as an endogenous control in all the qPCR experiments. 18S rRNA was used to normalize the mRNA levels of genes of interest before the comparison between different samples by the real time PCR. Housekeeping genes are stable, expressed in the cells and tissues of interest that do not show changes under the experimental conditions. Data was analysed by using the RQ Manager Version 1.2.1 (Applied Biosystems). Cycle threshold (CT) values were obtained at the end of qPCR for target genes from different experimental conditions. Mean CT values were calculated from the obtained CT values for triplicates. The relative expression for target genes was calculated by Relative quantification (RQ) using the equation  $RQ=2^{-\Delta\Delta Ct}$

### 2.19.8 Multiplex analysis

Multiplex assay was performed to determine the expression of multiple proteins with in a sample. As described above in the experiment of 2.13.1 and 2.17 (material and method section), the samples supernatants were collected from the phagocytosis assays after 24 hour and 48 hour of treatments to THP1-cells to determine secreted cytokines (IL-6, IL-10, IL12p40, IL12p70, IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-17A, IL-4, TNF- $\beta$ ), chemokines (MCP-3, MDC, Eotaxin, Fractalkine, GRO, MCP-1), growth factors (IL-2, FGF, G-CSF, GM-CSF, IL-4, VEGF) and other ligands and receptors (IFN- $\gamma$ , FLT-3L, IL-1RA). MagPix Milliplex kit (EMD Millipore) was used to measure cytokine response following the manufacturers protocol. 25  $\mu$ L of assay buffer was added to each well of a 96-well plate, followed by the addition of 25  $\mu$ L of standard,

controls or supernatants of cells treated with *M. bovis* BCG in the presence or absence of rfBC and complement. 25  $\mu$ L of magnetic beads coupled to analytes of interest were added in each well, and incubated for 18 hours at 4<sup>0</sup>C the 96-well plate was washed with the assay buffer and 25  $\mu$ L of detection antibodies were incubated with the beads for 1 hour at room temperature. 25  $\mu$ L of Streptavidin-Phycoerythrin was then added to each well and incubated for 30 mins at room temperature shaking at 750 rpm. Following a washing step, 150  $\mu$ L of sheath fluid was added to each well and the plate was read using the Luminex Magpix instrument. Assays were conducted in duplicate.

### **2.20 Statistical analysis**

Analysis of data for statistical significance was conducted using GraphPad Prism 5 for Windows (GraphPad Software, Inc). Statistical analyses were made using 2-way ANOVA for mRNA expression data and a 1-way ANOVA for the multiplex data. *p*-value of less than or equal to 0.05 was considered to be significant and. P values <0.05 were considered statistically significant represented with an asterisk (\*) on the graphs, unless otherwise stated (ns; nonsignificant). *p*-value of 0.01 to 0.001 represented by two asterisks (\*\*).

**Chapter 3: Purification and characterization of recombinant fragment of bovine conglutinin (rfBC) expressed in *Escherichia coli* and its impact on the growth of *M. smegmatis*.**

### 3.1 Introduction

Conglutinin synthesised in the liver (Holmsky *et al.*, 1992) and is a calcium dependent lectin, which occurs frequently in animals yet also found in chickens (Hogenkamp *et al.*, 2006) and fish (Nakao *et al.*, 2006).

The first aim was to express, purify and characterized the rfBC. This chapter describes the expression of the rfBC in *E.coli*, BL21 (DE3) pLysS (prokaryotic expression system). rfBC consist of neck and CRD region (conglutinin-neck-CRD) has been expressed and purified successfully. The recombinant protein denatured and purified using maltose-agarose affinity chromatography. Protein purification was confirmed by SDS-PAGE analysis. ELISA and western blot were performed to detect the rfBC presence in the purified samples. This recombinant protein, which is formed of neck and CRD region, was found to form trimers. Binding of rfBC was observed on *M. smegmatis* (non-pathogenic model of mycobacteria).

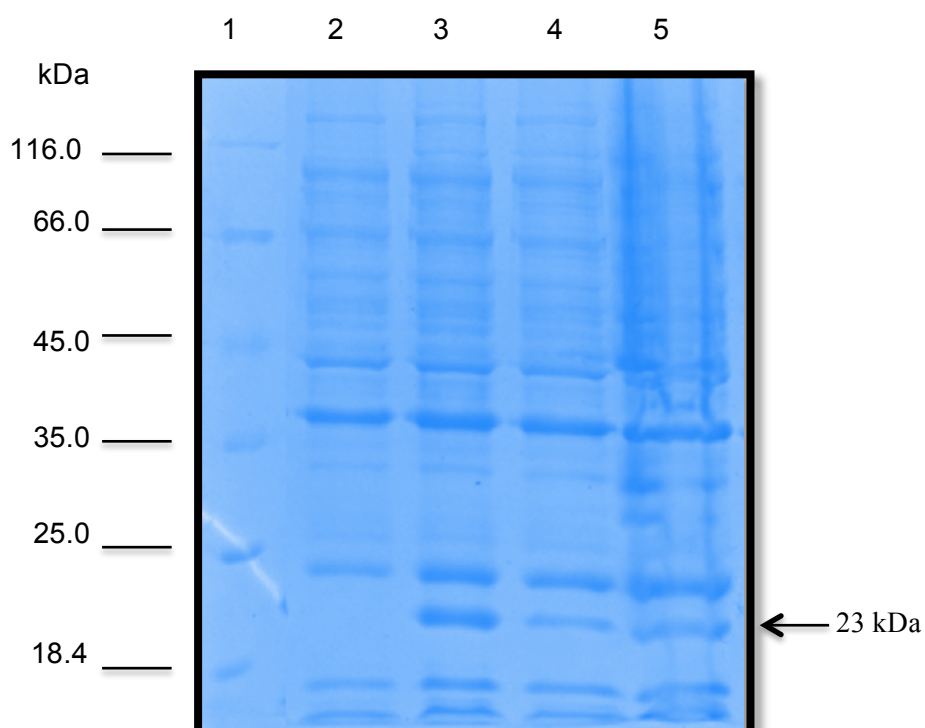
The second aim in this study was to investigate the following roles of rfBC on *M. smegmatis*: 1. Binding of rfBC with *M. smegmatis* (non-pathogenic model of mycobacteria). 2. Direct effect of rfBC on the growth of *M. smegmatis*. 3. Effect of rfBC on the phagocytosis of *M. smegmatis* by the THP-1 cells.

In this chapter MBP (Maltose binding protein) was expressed in *E.coli*, BL21 (DE3) pLysS and purified by affinity chromatography. The purpose of MBP production was to use it as a control protein in the characterisation and immunological assays.

## 3.2 Results

### 3.2.1 Expression and purification rfBC in *E. coli* (BL21 (DE3) pLysS strain)

rfBC was expressed in *E. coli* and purified by using affinity chromatography. Pilot expression was performed to check the expression of different clones after transformation. Visible protein bands at 23kDa showed that transformation was successful (Figure 3.1). Three transformed random colonies were picked up for further expression analysis. Colony number one (lane 3) showed higher yield of rfBC expression as compared to the other expressed clones analysed.

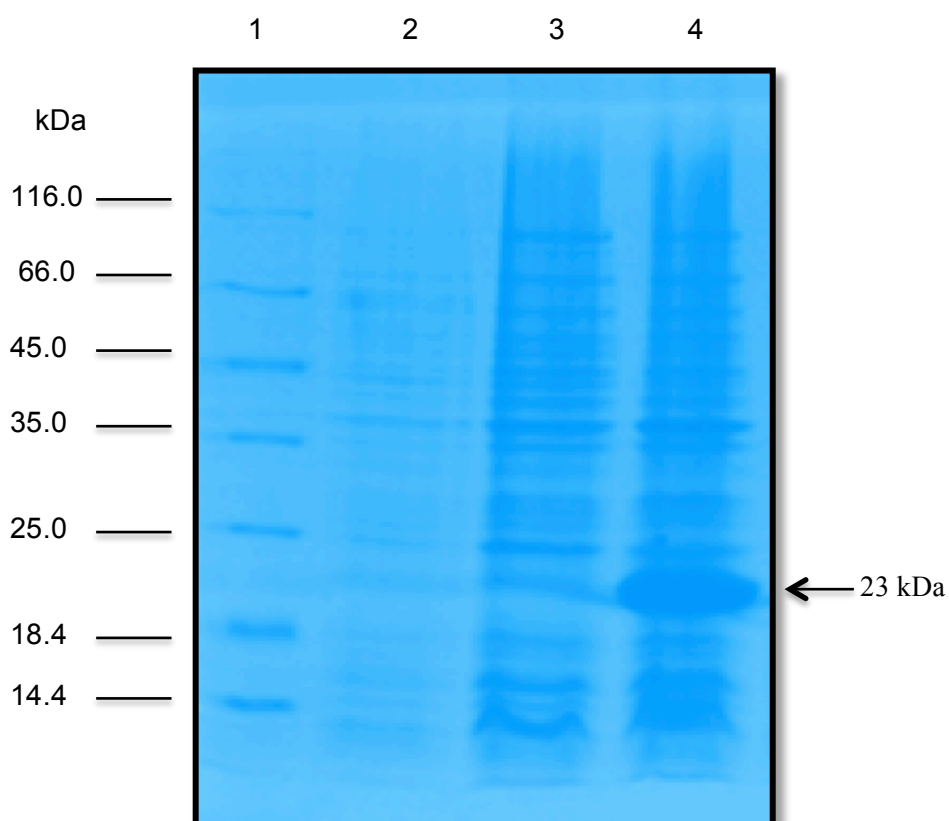


**Figure 3.1: Pilot expression of rfBC in *E. coli* BL21 (DE3) pLysS.** Lane 1 is the protein marker, Lane 2 is uninduced sample, and Lane 3,4 and 5 are IPTG induced cultures from colony 1, 2 and 3 respectively. Protein bands appear in all of the induced cultures at 23 kDa. rfBC expression is more prominent in colony 1 (Lane 3). Samples were run on a SDS-PAGE (12% w/v gel) followed by Coomassie staining.



### 3.2.2 Large scale expression of rfBC

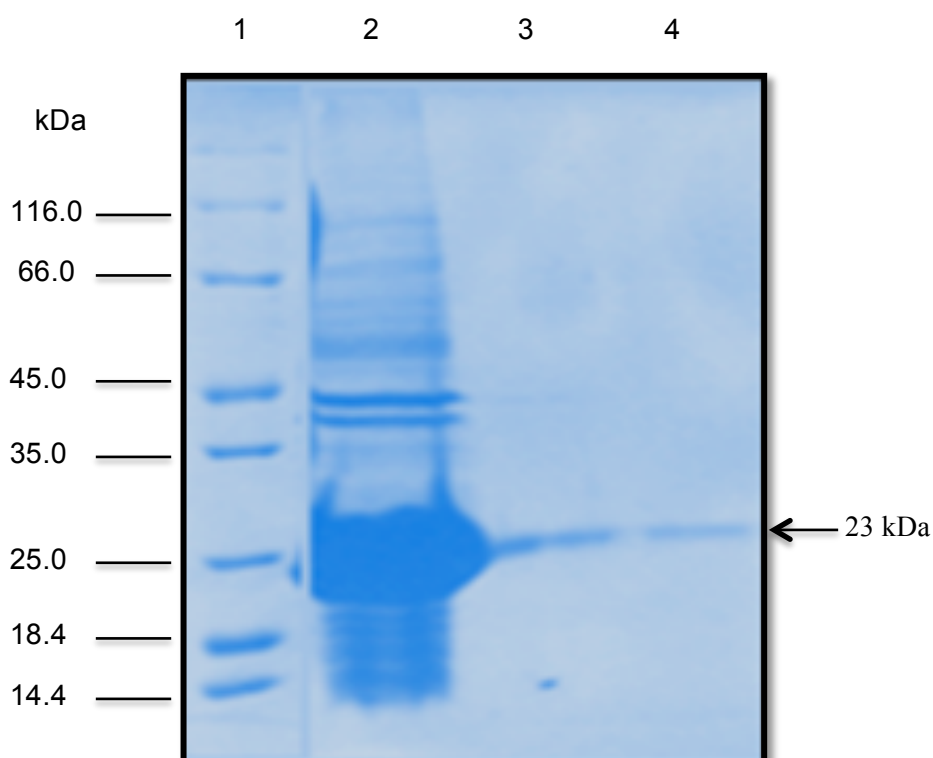
Large-scale expression was performed to produce a high yield of rfBC in order to perform the further experiments in this thesis. Colony 1 from pilot expression (Figure 3.1) was selected to inoculate in large-scale volume of the media and induced with IPTG to induced expression of rfBC (Figure 3.2).



**Figure 3.2: Large-scale expression of rfBC.** rfBC expression is analysed from the BL21 (DE3) pLysS colony 1. The samples were run on 12% SDS-PAGE (12% w/v gel). Lane 1 contained protein marker, Lane 2: un-induced sample, Lane 3: un-induced sample incubated 3 hours and Lane 4: Induced sample. The protein band appeared in lane 4 at molecular weight 23 kDa is the result of expression induction using IPTG and all the other bands are non interested proteins.

### 3.2.3 Purification of rfBC

The rfBC was purified from large-scale expressed cell lysate. SDS-PAGE showed that protein is present in the inclusion bodies in the cell pellet (Figure 3.3, lane 2). The cell lysate was lysed and sonicated. rfBC was recovered from inclusion bodies after denaturing and refolded by dialysis against decreasing concentrations of urea-TBS buffer. Protein was purified by ion exchange and affinity chromatography (Figure 3.3, lane 3 and 4).



**Figure 3.3: rfBC purified fractions.** Lane 1 contains the protein marker, Lane 2: Sample after lysis and sonication from cell pellet showing presence of rfBC in inclusion bodies, Lane 3 and 4 are purified rfBC (loaded in duplicate) at 23kDa expected band. The rfBC purified by ion exchange and affinity chromatography. Samples were run on a SDS-PAGE (12% w/v gel) followed by Coomassie staining.

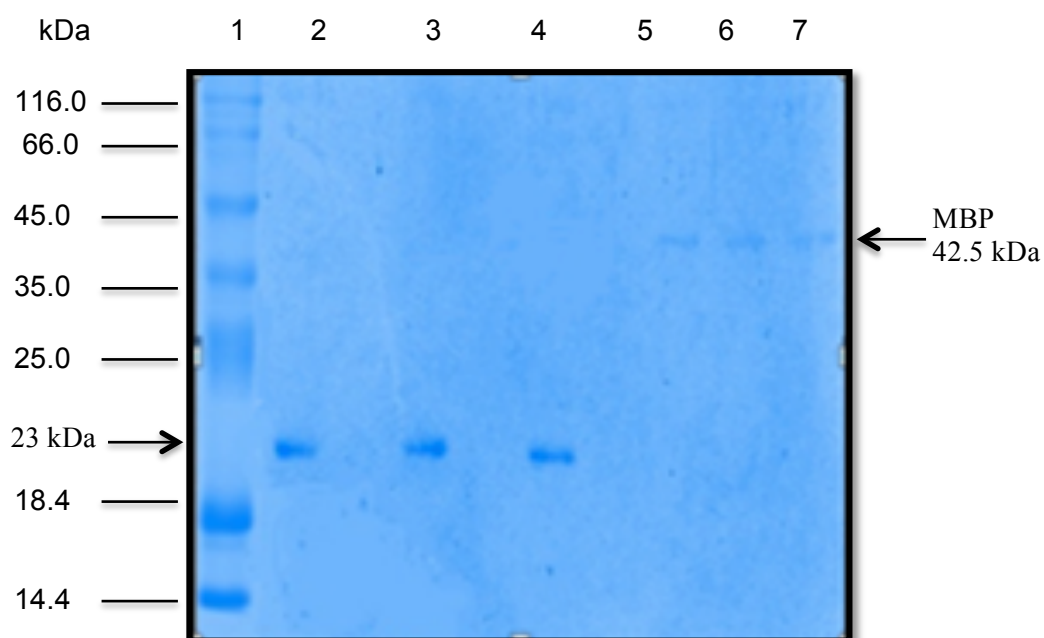
#### **3.2.4 Western blot and ELISA analysis of rfBC for confirmation of protein by anti-conglutinin.**

Western blotting was used to identify the rfBC. Purified rfBC is separated by SDS-PAGE and then transferred electrophoretically onto a nitrocellulose membrane. rfBC was identified by probing with rabbit anti bovine conglutinin.

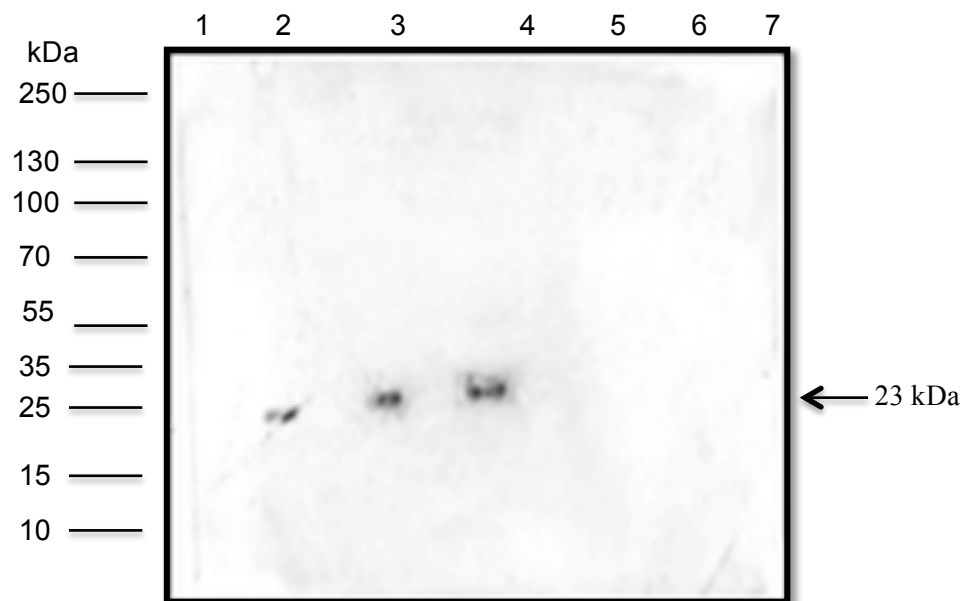
ELISA was also carried out to confirm the presence of rfBC in purified fractions. The results identified that rabbit anti bovine conglutinin monoclonal antibody bound with rfBC. There was no binding observed with the negative control protein (mannose binding protein) both in Western blot analysis (Figure 3.4B) and very little in ELISA assays (Figure 3.5).

### 3.2.4.1 Western blot analysis

rfBC is separated on SDS-PAGE for western blot analysis. Maltose binding protein (MBP) was used as a control protein. Three concentrations 5 $\mu$ g/ml, 10 $\mu$ g/ml and 20 $\mu$ g/ml of rfBC and MBP were loaded on to the gel to confirm the presence of rfBC in the purified samples (Figure 3.5 A). Following SDS-PAGE, primary and secondary antibodies applied to the samples. Western blot analysis confirms the identity and purity of rfBC using rabbit anti-bovine conglutinin. Western blotting with anti-MBP (not shown) confirmed no reaction with rfBC and reaction with 42.5 kDa MBP



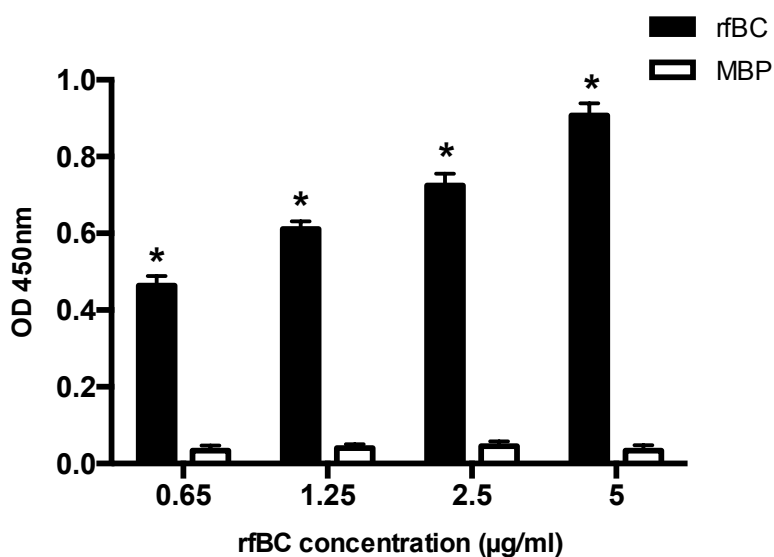
**Figure 3.4 A: SDS-PAGE gel of rfBC and MBP for western blot analysis.** Lane 1 contains the molecular mass standards (protein marker), lane 2 rfBC 5 $\mu$ g, lane 3 rfBC 10 $\mu$ g, lane 4 rfBC 20  $\mu$ g, lane 5,6 and 7 loaded with MBP 5 $\mu$ g, 10  $\mu$ g and 20  $\mu$ g respectively. Samples were run on 12% SDS-PAGE gel. MBP used as a negative control.



**Figure 3.4 B: Western blot analysis of rfBC for confirmation of proteins by anti- conglutinin.** The immunoblot shows three different concentration of rfBC that have been loaded. Lane 1 contains rainbow protein marker, lane 2, 3 and 4 with rfBC 5 $\mu$ g, 10 $\mu$ g and 20  $\mu$ g, lane 5,6 and 7 are loaded with MBP 5 $\mu$ g, 10  $\mu$ g and 20  $\mu$ g. MBP was used as a negative control. The gel was immunostained rabbit anti-bovine conglutinin antibody that identified rfBC at 23kDa.

### 3.2.4.2 ELISA to confirm the rfBC presence in purified fractions

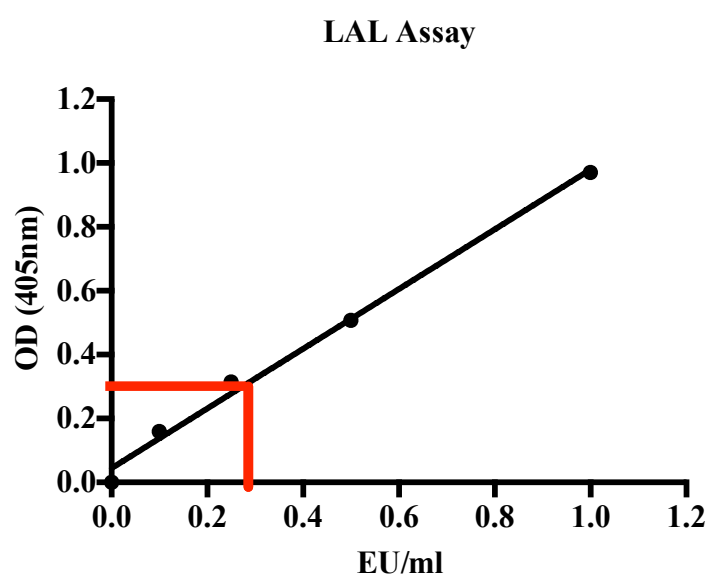
ELISA was performed to detect the rfBC presence in the purified fraction. ELISA plate was coated with different dilutions 0.65  $\mu\text{g}$ , 1.25  $\mu\text{g}$ , 2.5  $\mu\text{g}$  and 5  $\mu\text{g}$  of rfBC and then rabbit anti-bovine conglutinin was added to the rfBC and secondary antibody used to detect the complex. MBP used as a control. We found the binding with anti-rfBC in all the concentrations but we could not find significant binding at any concentration of MBP (Figure 3.5). This shows us rfBC is present in the purified samples.



**Figure 3.5: ELISA data confirmed presence of rfBC in the purified fractions.** Different serial dilutions of rfBC and MBP (0.65  $\mu\text{g}$ , 1.25  $\mu\text{g}$ , 2.5  $\mu\text{g}$  and 5  $\mu\text{g}$ ) were treated with monoclonal anti-rfBC. Samples were read at 450nm. Each histogram represents the average of 3 independent experiments. Error bars represent the SEM. There is a significance difference established MBP and rfBC (conglutinin). P value analyse by applying unpaired one-way ANOVA test (\* $p < 0.05$ ).

### 3.2.5 Endotoxin level measurement (LAL assay) of rfBC

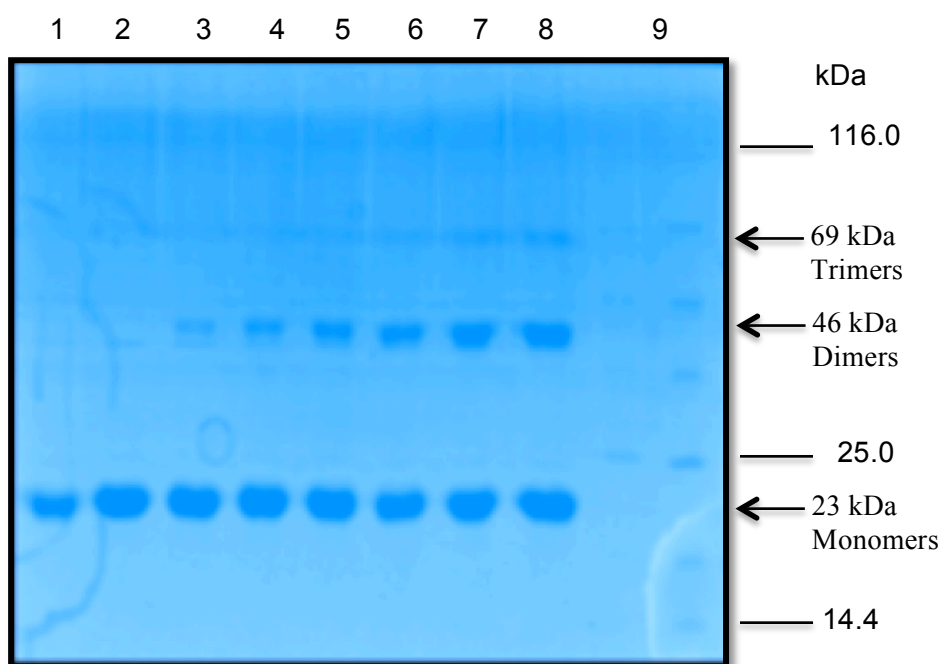
The Limulus amoebocyte lysate (LAL) assay was performed to measure the endotoxin level in the purified rfBC. Chromogenic LAL endotoxin assay kit (Genscript) was used to carry out the test. The endotoxins activate Factor C proteolytic activity present in LAL, which is photometrical, measured using chromogenic substrate at optical density ( $OD_{405}$ ). The endotoxin level in rfBC was 0.23 EU/ml and assay results were linear (Figure 3.6). One EU is equal to 100pg of *E. coli* lipopolysaccharide.



**Figure 3.6:** LAL assay to measure endotoxin levels in the purified rfBC. Each data point shows the optical density for triplicate data from different concentrations of endotoxin solution. The endotoxin level in purified rfBC were found to be ~0.23 pg/mg.

### 3.2.6 Cross-linking of rfBC

To verify that rfBC was correctly folded and formed a homotrimer in solution, chemical cross-linking using BS3 was performed on the purified protein. rfBC formed trimers in a time and BS3 concentration-dependant manner. It has been confirmed that rfBC formed the dimers and trimers same way as in native form (Figure 3.7).

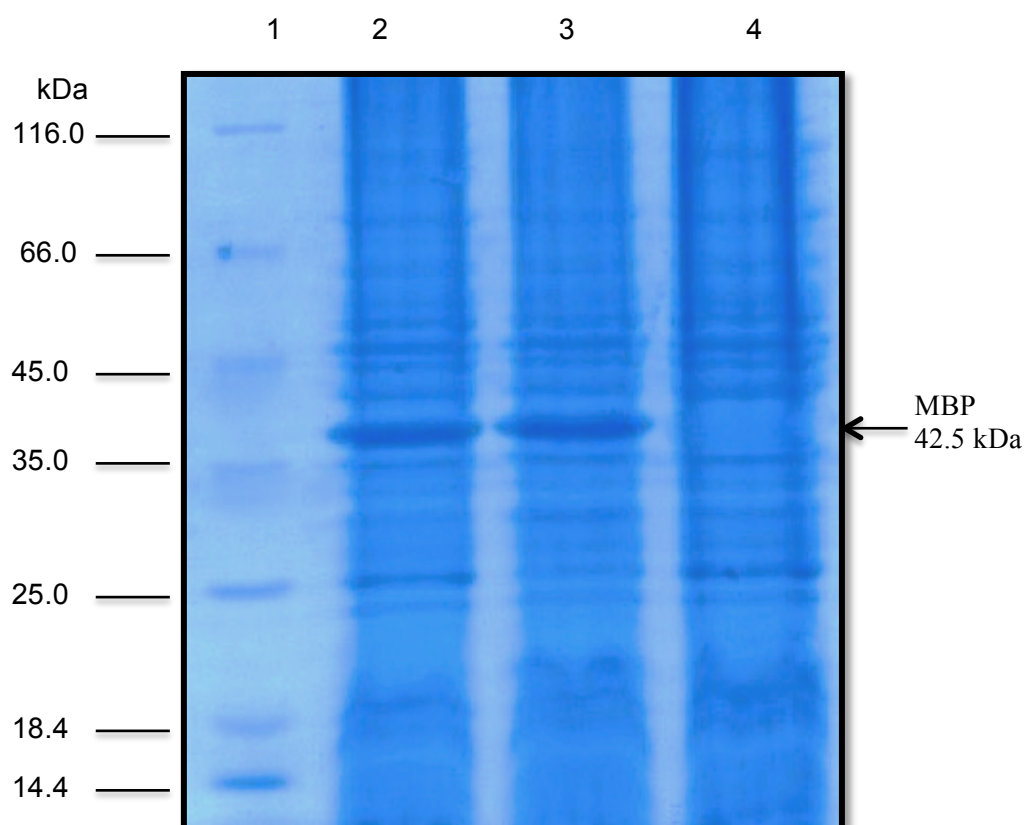


**Figure 3.7: Chemical cross-linking of purified rfBC to show that it could form dimeric and trimeric structures.** 20  $\mu\text{g/ml}$  of rfBC were incubated with 1mM BS<sup>3</sup> (bis(sulfosuccinimidyl)suberate) for 0 min (lane 1), 1 min (lane 2), 2 min (lane 3), 4 min (lane 4), 8 min (Lane 5), 16 min (Lane 6), 32 min (lane 7), 64 min (lane 8). Protein marker (lane 9). Samples were resolved on a SDS-PAGE (12% w/v gel) followed by Coomassie staining and show dimers at 46 kDa and trimers at 69 kDa.

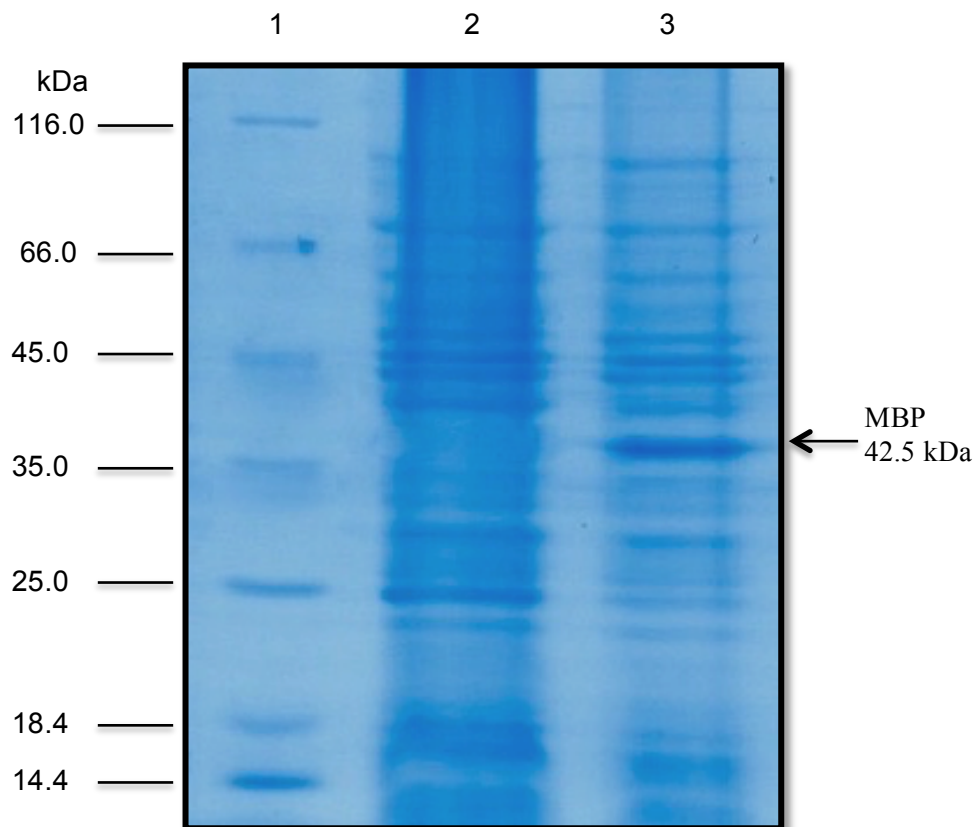


### 3.2.7 Expression and purification of maltose binding protein (MBP) in *E. coli* (BL21 (DE3) pLysS strain)

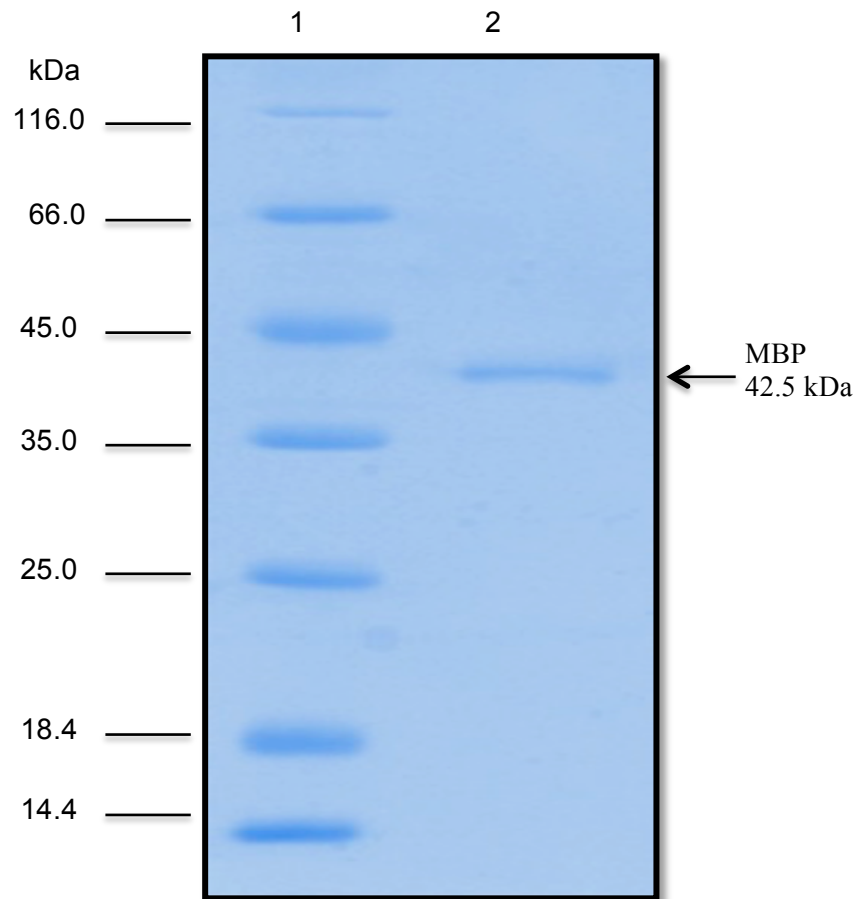
Maltose binding protein was expressed in *E. coli* and purified by using amylose resin column. Pilot expression confirmed the successful transformation of MBP plasmid in to the *E. coli* (Figure 3.8A). One of the colonies from pilot expression was selected to produced MBP at large scale. In large scale clone expressed the MBP protein very well (Figure 3.8B). MBP was purified from the cell lysates by passing through amylose resin column. The eluted fraction was characterised on 12% SDS-PAGE, shown the expected band at 42.5 kDa (Figure 3.8C).



**Figure 3.8A: Characterisation of MBP produced during pilot expression.** Clear MBP band shown in both clones of the MBP and there is no band in the uninduced sample. Lane 1 contains protein marker. Lane 2 and 3 were loaded with induced clones of MBP and bands appeared at 42.5 kDa in both clones. Lane 4 comprised of an un-induced sample. Samples were run on a SDS-PAGE (12% w/v gel) followed by Coomassie staining.



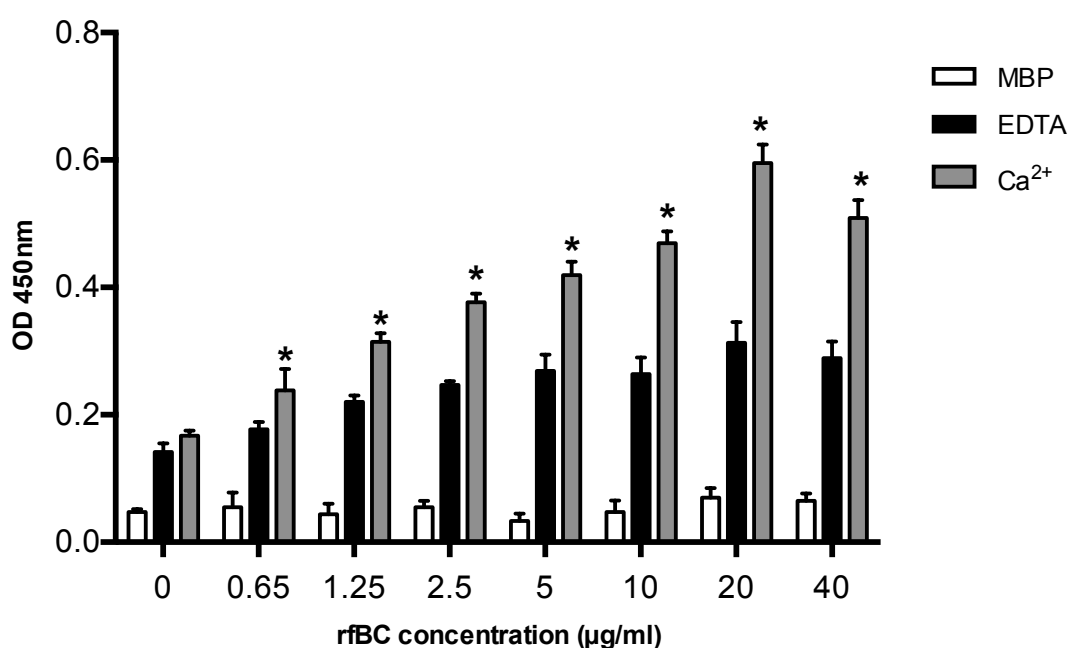
**Figure 3.8 B: Characterisation of MBP produced during large-scale expression.** MBP band shown in the induced sample and there is no band in the uninduced sample. Lane 1 contains protein marker. Lane 2 with un-induced sample and Lane 3 loaded with induced sample. MBP appeared at 42.5 kDa in induced colony. Samples were run on a 12% SDS-PAGE (12% w/v gel) followed by Coomassie staining.



**Figure 3.8 C: Purified fraction of maltose binding protein (MBP).** Purified fraction of MBP characterised on 12% SDS-PAGE. Purified MBP migrated at a molecular weight of 42.5 kDa. Lane 1 contains protein marker and lane 2 with MBP purified fraction. Samples were run on a 12% SDS-PAGE (12% w/v gel) followed by Coomassie staining.

### 3.2.8 Binding of rfBC to *M. smegmatis* in the presence of calcium and EDTA

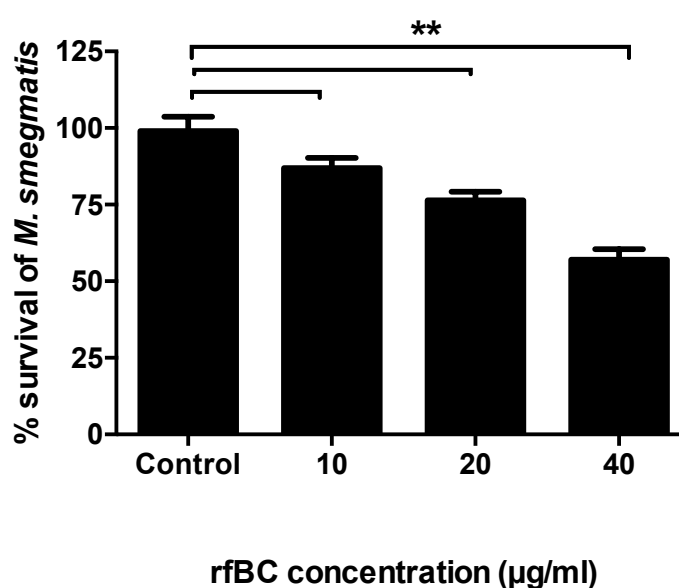
This study was performed to investigate the binding of rfBC to *M. smegmatis* and also to see either this binding is calcium dependent. Higher binding observed at higher concentration of rfBC and also it was confirmed that strong binding observed in the presence of calcium components. There was less binding observed in the presence of EDTA (Figure 3.9).



**Figure 3.9: Binding of rfBC to *M. smegmatis*.** No rfBC and MBP (Maltose binding protein) were used as a negative control and triplicate the condition to calculate the average for more accuracy. Data showed us that the binding was calcium dependent and maximum binding observed with 20 µg rfBC concentration. Each histogram represents the average of 3 independent experiments. Error bars represent the SEM. There is a significance difference established between *M. smegmatis* binding with rfBC in EDTA buffer and *M. smegmatis* binding with rfBC in Ca<sup>2+</sup> buffer. Error bars represent the standard error of the mean (SEM). P value analysis by applying unpaired one-way ANOVA test (\*p<0.05).

### 3.2.9 Direct effect of rfBC on *M. smegmatis* growth

In this study, we found that rfBC directly inhibited the growth of *M. smegmatis*. Three concentrations 10 µg, 20 µg and 40 µg of rfBC incubated with mycobacteria for 2 hours (1 hour at 37<sup>0</sup>C and 1 hour at 4<sup>0</sup>C) and 0 µg rfBC was used as a control (Figure 3.10). rfBC inhibited the growth of *M. smegmatis*. Growth was inhibited 13% with 10 µg/ml rfBC, 24 % with 20 µg/ml rfBC and 40 µg/ml of rfBC inhibited the growth of *M. smegmatis* directly by 44% and the results are statistically significant. Three independent experiments shown the same trend of growth inhibition (Figure 3.10).

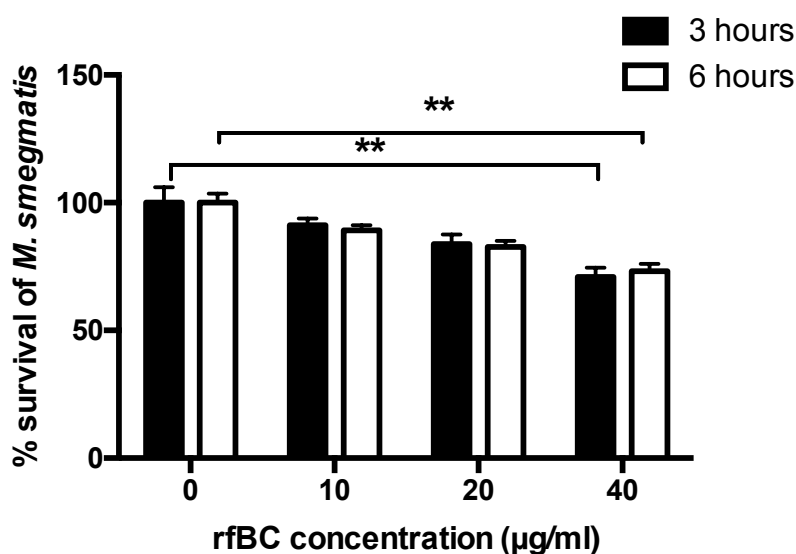


**Figure 3.10 : Direct Effect of rfBC on *M. smegmatis* growth.** Different concentration of rfBC 10 µg, 20 µg and 40 µg were added to *M. smegmatis*. *M. smegmatis* were incubated with and without rfBC for 2 hours. Bacterial colony forming units were obtained and counted. The percentage difference in growth for each rfBC concentration was calculated with respect to untreated *M. bovis* BCG as 100% (corresponding to  $6 \times 10^4$  cells). The experiment was performed in triplicate for each rfBC concentration and was repeated 3 times. There is a significance difference established between un-treated samples (*M. smegmatis* only) and *M. smegmatis* treated with 40 µg rfBC. Error bars represent the standard error of the mean (SEM). P value analyse by applying unpaired one-way ANOVA test (\* $p < 0.05$ , \*\* $p \leq 0.01$ ).

### 3.2.10 Effect of rfBC on uptake of *M. smegmatis* by THP-1 cells

The effect of rfBC on uptake of *M. smegmatis* by THP-1 cells was studied after 3 and 6 hours treatment. In this study results shown that rfBC reduced the uptake with a *M. smegmatis*, reduction of uptake was observed 9 % with 10 µg/ml rfBC, 17 % with 20 µg/ml rfBC and 27% reduction of uptake of *M. smegmatis* treated with 40µg rfBC by THP-1 cells as compared to untreated mycobacteria for 3 hours.

While after 6 hours incubation of *M. smegmatis* with THP-1 cells, reduction in uptake was observed 11 % with 10 µg/ml rfBC, 17 % with 20 µg/ml rfBC and 26% reduction of uptake of *M. smegmatis* treated with 40µg rfBC by THP-1 cells as compared to untreated mycobacteria which is nearly similar to the 3 hours results (27%). The results are statistically significant ( $p=0.05$ ) with only 40µg/ml rfBC for 3 and 6 hours.



**Figure 3.11: Effect of rfBC on the uptake of *M. smegmatis* by THP-1 cells.** Three concentration (10µg, 20µg, 40µg) of rfBC were incubated with *M. smegmatis* for two hours. rfBC coated *M. smegmatis* were treated to THP-1 cells for 3 and 6 hours to study the effect of rfBC. Bacterial colony forming units were obtained and counted. The percentage difference in growth for each rfBC concentration was calculated with respect to untreated *M. bovis* BCG as 100% (corresponding to  $6 \times 10^4$  cells). The experiment was performed in triplicate for each rfBC concentration and was repeated 3 times. There is a significance difference established between un-treated samples (*M. smegmatis* only) and *M. smegmatis* treated with 40 µg rfBC for 3 and 6 hours. Error bars represent the standard error of the mean (SEM). P value analyse by applying unpaired one-way ANOVA test (\* $p<0.05$ , \*\* $p\leq 0.01$ ).

### 3.3 Discussion

In this study, rfBC protein consisting of residues 197-351 of the native protein was successfully expressed (Figure 3.1, lane 3-5 and Figure 3.2, lane 4). The residues 197-224 (28 amino acids) represent  $\alpha$ -helical neck region and residues 225-351 (127 amino acids) make the long C-terminal CRD region of the naturally produced bovine conglutinin (Wang *et al.*, 1995). The rfBC was expressed by using the T7 promoter expression system *E.coli*, BL21 (DE3) pLysS that produced a large amount of protein. It has been observed that rfBC formed inclusion bodies (Figure 3.3, lane 2). In order to denature and refolding of insoluble form of rfBC it was dialysed against different concentration of urea buffer. The expressed rfBC protein consisting of neck-CRD was purified by ion exchange and affinity chromatography and gave a major band of 23 kDa on SDS-PAGE (Figure 3.3, lane 3), which is similar in molecular size to that of rfBC (Wang *et al.*, 1995).

The rfBC protein was made free of endotoxin before use in further experiments, as endotoxin can interfere in the experiments by causing the activation of macrophages (Martinez *et al.*, 2008). LAL assay was used to measure the concentration of endotoxin in the expressed rfBC. The endotoxin level was found to be less than 1EU/ml (Figure 3.6). The purified rfBC protein was characterised by performing western blot and ELISA by using rabbit anti-rfBC IgG as a probe, MBP used as a control and the rfBC specific antibody showed no binding to this protein.

Native bovine conglutinin monomers can combine to form a trimeric structure that is stabilised by inter-monomer covalent disulphide bridges, binding cysteine residues in N-terminals or in collagen domain (Hoppe and Reid, 1994). rfBC has been expressed as a neck-CRD region, in order to investigate whether this protein can also form dimer and trimer to stabilise the rfBC structure. Cross linking of the rfBC protein via BS3 linker showed its ability to form dimers and trimers (Figure 3.7), suggesting that conglutinin-neck-CRD have potential to form oligomers. Dimers and trimers increase the affinity of conglutinin protein to bind to its receptors. As natural bonds between the polypeptide chains are weaker and while running the sample on SDS-PAGE treated with buffers and heat treatment can easily break weak bonds so BS3 cross linker used to strength the bond to easily visible to characterise the rfBC presence in the solution.

In this study, recombinant fusion mannose binding protein (MBP) was expressed and purified. MBP was produced using *E. coli*, BL21 (DE3) pLysS expression system. The MBP was purified by affinity chromatography using amylose resin column and a major band appeared at 42.5 kDa on SDS-PAGE (Figure 3.8 C, lane 2). MBP was produced to be used as a negative control for rfBC protein in this study.

Conglutinin is structurally similar to another important member of collectin family lung surfactant protein D (SP-D) (Lu *et al.*, 1993) and SP-D is able to target glycol conjugates on pathogens, preventing infection by stimulating phagocytes to eliminate pathogens and also by regulating immune responses (Ferguson *et al.*, 1999). It is thought that the additional bovine collectin is derived from gene duplication events of an ancestral bovine SP-D gene. However, unlike other members of collectin family, conglutinin is exclusively found in cattle (Holmskov, 2000). Unlike SP-D, the biological function of conglutinin is not well understood. Conglutinin is synthesised in the liver and found in serum at approximate concentrations of 12 µg/ml (Holmskov *et al.*, 1998). Conglutinin has also been found in the spleen, tonsils and lymph nodes (Holmskov *et al.*, 1992). It is currently not known whether conglutinin is synthesised locally e.g. lungs as a result of respiratory infection. A limited number of studies have shown that conglutinin does bind to microorganisms. Conglutinin has been shown to bind to viruses (bovine herpes virus 1, influenza A virus and Nebraska calf diarrhoea virus) and can aggregate, enhance attachment and enhance phagocytosis and killing (Reading *et al.*, 1998; Hartshorn *et al.*, 1993). Conglutinin has also been shown to bind with yeast cells such as *S. cerevisiae* and *C. albicans* in the presence of Ca<sup>2+</sup> ions (Dec *et al.*, 2012). It has been shown that native conglutinin and rfBC binds to LPS (lipopolysaccharides) of Gram-negative bacteria, such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* (Wang *et al.*, 1995). However, a detailed evaluation of the role of conglutinin in bovine infection and immunity is yet to be carried out.

The collectins trimer structure plays a vital part in the strong interaction to the carbohydrate-containing microorganism and present them to immune cells (Hakansson and Reid 2000). Conglutinin is composed of 4 trimeric units (Hansen *et al.*, 2002). Conglutinin has the ability to bind with *N*-acetyl glucosamine, mannose, and fucose residues (Loveless *et al.*, 1995), and can also bind with zymosan (Andersen *et al.*,



1992). It has also been reported that conglutinin binds to zymosan and iC3b (complement fragment) in a calcium dependent manner (Dec and Wernicki, 2006). In this study, conglutinin binding to *M. smegmatis* was investigated. In this study we show for the first time that rfBC can bind to the surface of *M. smegmatis* and this binding is dependent on  $\text{Ca}^{2+}$ , as it is C-type lectins (Figure 3.9). In contrast, binding in the presence of EDTA was observed 3 to 4-fold less, while almost no binding was observed with maltose-binding protein (MBP) (Figure 3.9). This shows that conglutinin is a calcium dependent lectin confirming previous findings (Friis *et al.*, 1991). A concentration of 20  $\mu\text{g/ml}$  of rfBC was enough to reach saturation of binding, which is almost similar to the approximate in vivo serum levels of rfBC in cattle of 12  $\mu\text{g/ml}$  (Holmskov *et al.*, 1998). The binding of rfBC with mycobacteria has not been reported before. However, it has been shown that SP-D two important ligands present on the outer surface of *M.tb* those are LAM and mannose capped LAM (ManLAM) (Ferguson *et al.*, 1999).

We also investigated the direct effect of rfBC on *M. smegmatis* growth. We observed the growth inhibition of *M. smegmatis* by rfBC in a dose dependent manner and the same trend of growth inhibition was observed in all the experiments performed. There is 13% reduction in growth observed for 10  $\mu\text{g/ml}$ , 24% for 20  $\mu\text{g/ml}$  and 44% for 40  $\mu\text{g/ml}$  of rfBC. The growth inhibitory effect at 40 $\mu\text{g/ml}$  of rfBC was statistically significant (Figure 3.10). The direct effect of rfBC on mycobacteria has not been studied previously. It has been reported that mice injected with conglutinin prolonged the survival of the mice after infection with *Salmonella typhimurium* (Friis *et al.*, 1991).

*M. smegmatis* was incubated with rfBC for 2 hours prior to treat with THP-1 cells. The infection with THP-1 cells after 3 and 6 hours shown to reduce the uptake of *M. smegmatis* coated with rfBC (Figure 3.11). There is 11% reduction in growth observed for 10  $\mu\text{g/ml}$ , 17% for 20  $\mu\text{g/ml}$  and 26 % for 40  $\mu\text{g/ml}$  of rfBC. This reduction in uptake of *M. smegmatis* by THP-1 cells was significant at 40 $\mu\text{g/ml}$  of rfBC (Figure 3.14). The reason for *M. smegmatis* minimal uptake could be rfBC is masking mycobacterial ligands and preventing uptake, possibly through mannose receptors present on macrophages. In one of the study it has been reported that SP-D most similar member of collectin family to the conglutinin also shown the less uptake of the

*M. tb* by macrophages by masking the mannose receptors those play a significant role in the phagocytosis of mycobacteria (Ferguson *et al.*, 1999).

**Chapter 4- The role of conglutinin (rfBC) in mycobacterial infection. Insights from interactions with *M. bovis* BCG**

## 4.1 Introduction

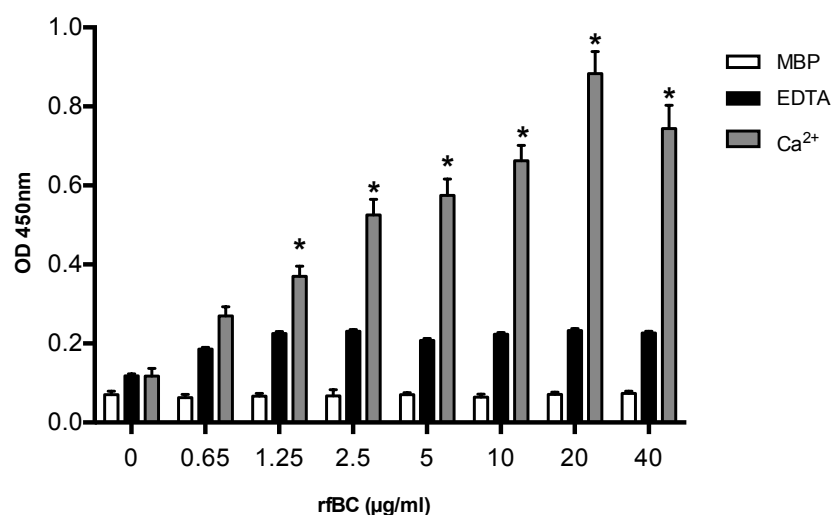
Bovine conglutinin is structurally very identical to Surfactant Protein D (SP-D). It has been known that SP-D interacts with *Mycobacterium tuberculosis*. *Mycobacterium tuberculosis* is very similar to *M. bovis*, the causative agent of bovine tuberculosis. We assume that due to the overall relevance between conglutinin and SP-D, conglutinin is likely to have a protective impact in bovine tuberculosis. This has never been studied before. Conglutinin mediates bactericidal activity toward Gram-negative bacteria in a system containing adherent leukocytes and complement (Friis-Christiansen *et al.*, 1990).

In this chapter, we investigated the potential of the host bovine protein (rfBC) for its role in mycobacterial infection. The aim was to investigate the immunological properties of the rfBC in vitro. In the previous chapter we established that rfBC binds with *M. smegmatis*, study model for mycobacterial species. In this study we used *M. bovis* BCG as a model organism for *M. bovis* and THP-1 macrophages as a model cell line for phagocytes. The first aim in this study was to establish the rfBC is a calcium dependent lectin to bind *M. bovis* BCG in presence of calcium. The second aim was to study the role of rfBC in host-pathogen interactions in bovine tuberculosis infection and investigating its potential as a therapeutic. For this study, we investigated following roles of rfBC on *M. bovis* BCG: 1. Direct effect of rfBC on *M. bovis* BCG. 2. To study whether rfBC bound to the surface of *M. bovis* BCG inhibits the phagocytosis of *M. bovis* BCG by THP-1 cells. 3. To investigate if rfBC regulates cytokine responses during phagocytosis of and how these may play a role in adaptive immunity in bovine tuberculosis.

## 4.2. Results

### 4.2.1: Binding of rfBC to *M. bovis* BCG

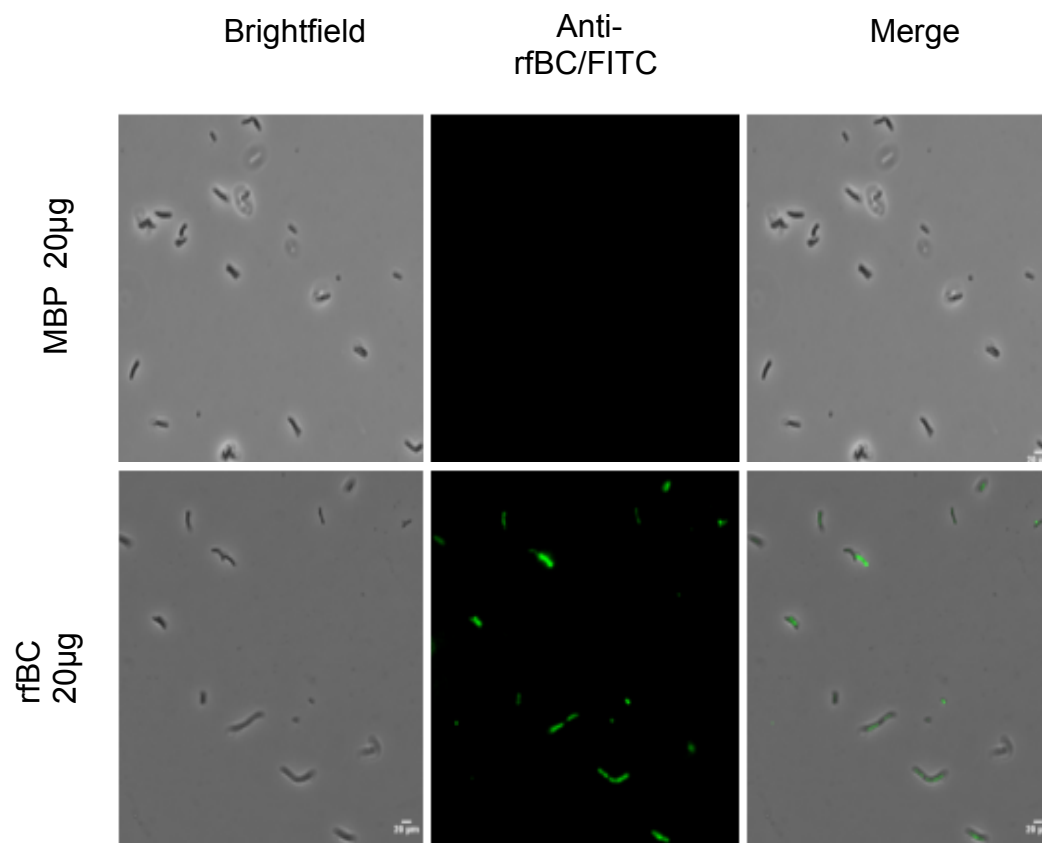
ELISA was performed to show the binding of different concentration of rfBC to *M. bovis* BCG as described in the material and method section (2.2). *M. bovis* BCG only and MBP used as a controls. Proteins were diluted in two different buffers (5 mM Ca<sup>2+</sup> and 5 mM EDTA), to study the calcium dependent property of rfBC in binding. Binding increased with increase concentration until 20 µg/ml of rfBC (Figure 4.1). It was observed that there is maximum binding at 20 µg/ml of rfBC and the rise in binding was dose-dependant. Significant binding was also observed only in the presence of Ca<sup>2+</sup> and was inhibited in the presence of EDTA. Binding in the presence of 5 mM EDTA was reduced 3 to 4-fold, whilst negligible binding was seen with MBP.



**Figure 4.1: Serial dilution of rfBC with *M. bovis* BCG in the presence of 5mM Ca<sup>2+</sup> and 5mM EDTA buffers.** rfBC binding to *M. bovis* BCG cells in the presence of Ca<sup>2+</sup> with EDTA and maltose-binding protein used as negative controls. Two-fold serial dilutions of rfBC were performed in 40mM Tris-HCl, 200mM NaCl, pH 7.4 containing 5 mM CaCl<sub>2</sub> or 10 mM EDTA and added to the mycobacteria and allowed to incubate for 2 h. This was followed by incubation with rabbit anti-rfBC antibody or mouse anti-MBP monoclonal antibody. Then, protein A-HRP was added and developed with the substrate 3, 3',5,5'-Tetramethylbenzidine (TMB) and the colour read at 450 nm. All assays were conducted in triplicate. There is a significance difference established between *M. bovis* BCG binding with rfBC in EDTA buffer and *M. bovis* BCG binding with rfBC in Ca<sup>2+</sup> buffer. Error bars represent the standard error of the mean (SEM). P value analyse by applying unpaired one-way ANOVA test (\*p<0.05).

#### **4.2.2 Binding of rfBC with *M. bovis* BCG by immunofluorescence microscopy**

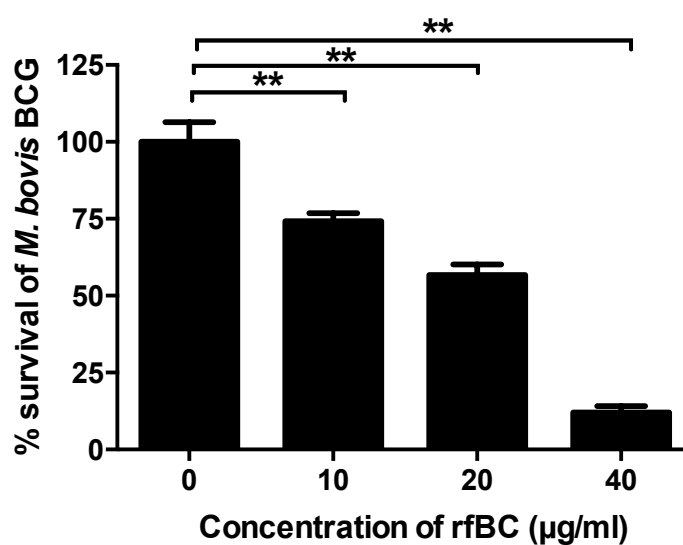
Immunofluorescence microscopy was performed to detect the binding of rfBC. In the previous binding experiment (Figure 4.1), we observed maximum binding on *M. bovis* BCG at 20 µg/ml of rfBC (Figure 4.1). Therefore this concentration was chosen to perform an immunofluorescence-binding assay for rfBC. Binding of rfBC was confirmed on *M. bovis* BCG. rfBC bound to *M. bovis* BCG in a calcium dependent manner (Figures 4.2, panels E and F), whilst no binding was observed with MBP, used as a negative control protein (Figures 4.2, panels E and F).



**Figure 4.2: Binding of rfBC with *M. bovis* BCG by immunofluorescence microscopy.** *M. bovis* BCG was incubated with 20 µg/ml of rfBC or 20 µg/ml of maltose binding protein (MBP) (used as a negative control). Rabbit anti-rfBC IgG (1:100 dilution) was used as the primary antibody and goat anti-Rabbit IgG (H+L) secondary antibody, FITC (1:500) was used for detection of bovine rfBC bindings with *M. bovis* BCG cells. rfBC-treated *M. bovis* BCG is shown by green fluorescence (panels E and F).

### 4.2.3 Direct effect of rfBC on *M. bovis* BCG growth

Here, it was demonstrated that surface-bound rfBC inhibited the growth of *M. bovis* BCG. Three concentrations of rfBC were investigated (10 µg, 20 µg and 40 µg/ml of rfBC) were incubated with mycobacteria for 2 hours (1 hour at 37°C and 1 hour at 4°C) and then cultured on agar plates for two weeks. The results revealed a dose-dependant trend that rfBC inhibited the growth of *M. bovis* BCG. Growth was inhibited by 26% with 10 µg/ml rfBC, 44 % with 20 µg/ml rfBC and 89% with 40 µg/ml rfBC, compared to *M. bovis* BCG only (control) (Figure 4.3).

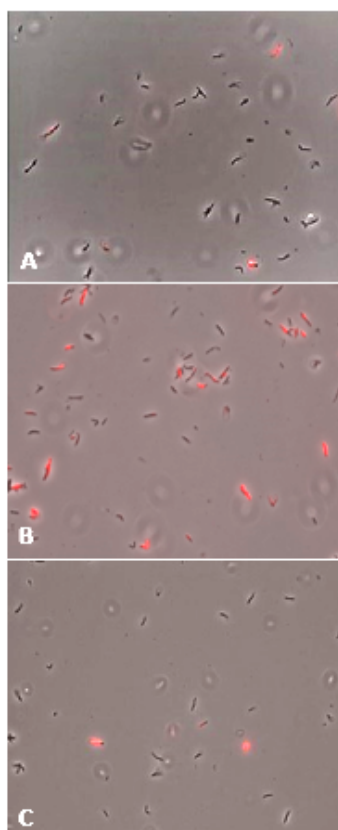


**Figure 4.3 : Direct Effect of rfBC to *M. bovis* BCG .** Different concentration of rfBC (10 µg, 20 µg and 40 µg/ml) were incubated with *M. bovis* BCG for 2 hours. Untreated *M. bovis* BCG was used as a negative control. Each bar represents the average of 3 experiments. There is a significance difference established between un-treated samples (*M. bovis* BCG only) and treated (*M. bovis* BCG + rfBC). Error bars represent the standard error of the mean (SEM). P value analyse by applying unpaired one-way ANOVA test (\*p<0.05, \*\*p<0.01) (n=3).



#### 4.2.4 Fluorescence microscopy to study the direct effect of rfBC on the *M. bovis* BCG.

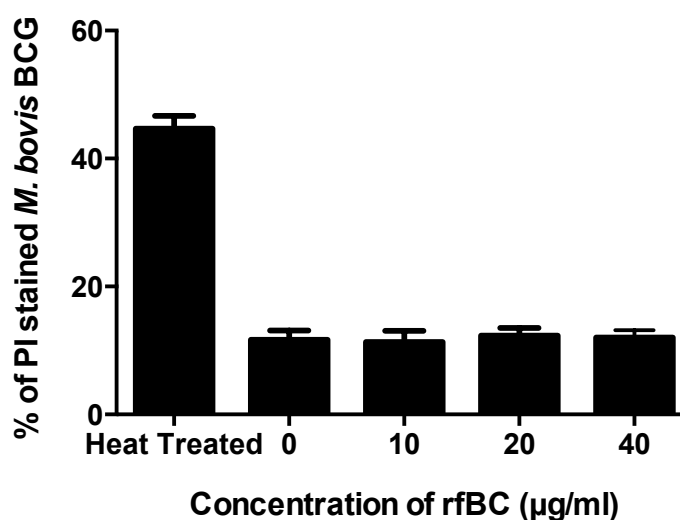
This experiment was performed to see whether rfBC lysed the cell membrane of the mycobacteria, which resulted in *M. bovis* BCG growth inhibition. We have not observed bacterial cell lyses in untreated *M. bovis* BCG (Figure 4.4, panel A) and rfBC- treated *M. bovis* BCG (Figure 4.4, panel C), as determined by propidium iodide (PI) staining. Heat-treated *M. bovis* BCG were used as a positive control for cell lysis (Figure 4.4, panel B). These results appear to suggest that rfBC may be of importance in the control of systemic mycobacterial infection.



**Figure 4.4: Fluorescence microscopy of *M. bovis* BCG stained with propidium iodide.** Experiment was performed to observe the bacterial cell lysis as a result of rfBC treatment. Impact of rfBC on the *M. bovis* BCG growth. *M. bovis* BCG cells were incubated for 2 h with 20 µg/ml of rfBC. *M. bovis* BCG cells were stained with 1 µg/ml Propidium Iodide. Untreated *M. bovis* BCG were used as a negative control (panel A); Heat-treated *M. bovis* BCG was used as a positive control for cell lysis (panel B); *M. bovis* BCG treated with 20 µg/ml rfBC (panel C).

#### 4.2.5 *M. bovis* BCG cells stained by Propidium Iodide (PI) after direct effect of rfBC.

In this study we showed the quantitative data of direct effect of rfBC on the *M. bovis* BCG cell membrane permeability and lysis (Figure 4.5). This data confirmed the qualitative data (Figure 4.4), that rfBC does not damage the mycobacterial cell membrane and that inhibition is not due to cell lysis. Here, we treated *M. bovis* BCG with different concentration (10  $\mu\text{g}$ , 20  $\mu\text{g}$  and 40  $\mu\text{g}/\text{ml}$ ) of rfBC, to see whether differing concentrations would affect the above observation. Heat-treated *M. bovis* BCG was used as a positive control and untreated *M. bovis* BCG was used as a negative control. The data is an analysis of an average of 6 microscopic fields of view.

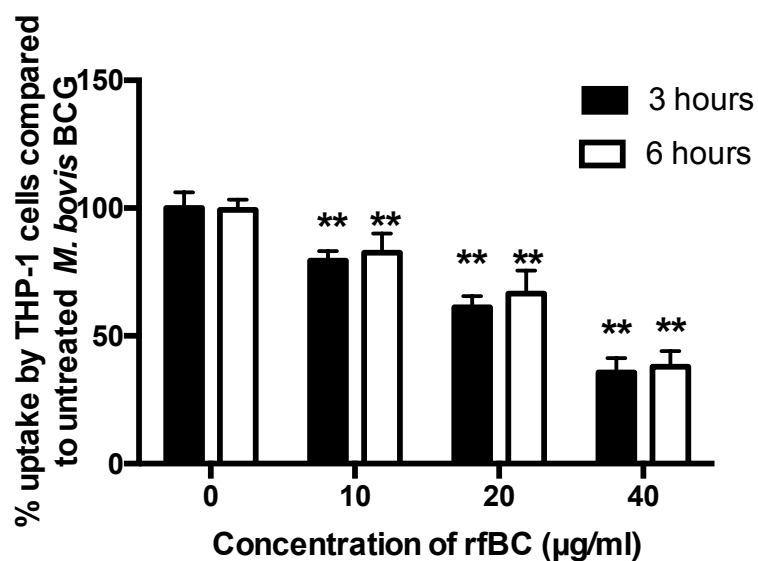


**Figure 4.5: Propidium iodide (PI) assay to assess bacterial cell lysis as a result of incubation with rfBC.** *M. bovis* BCG cells were incubated for 2 h with 0  $\mu\text{g}/\text{ml}$ , 10, 20 and 40 $\mu\text{g}/\text{ml}$  of rfBC. PI was added, and cells spotted onto glass slides and analysed using fluorescence microscopy. Fluorescent *M. bovis* BCG cells were counted from each sample and the total percentage of PI stained cells calculated for each rfBC concentration. Error bars represent  $\pm 2$  standard deviations. The assay was performed in triplicate. A one-way ANOVA test was performed to determine significant differences in PI staining from heated-treated *M. bovis* BCG versus treatment with rfBC at the different concentrations. All comparisons were significant ( $p \leq 0.05$ ), unless where shown (ns: not significant,  $p > 0.05$ ).

#### 4.2.6 Effect of rfBC on uptake of *M. bovis* BCG by THP-1 cells

THP-1 cells were incubated with *M. bovis* BCG treated with three concentration (10 µg, 20 µg, 40 µg) of rfBC for 3 and 6 hours (Figure 4.6). It was observed that uptake of *M. bovis* BCG treated with rfBC was decreased by THP-1 cells after 3 and 6 hours of incubation. Three independent experiments were performed to study the effect of rfBC on uptake of *M. bovis* BCG by THP-1 cells. All the experiments showed the same trend with a decrease of *M. bovis* BCG uptake by THP-1 cells in the presence of 10µg, 20µg, and 40µg/ml rfBC after 3 hours of the incubation. The following reduction of uptake of *M. bovis* BCG was observed: 19% at 10 µg /ml rfBC, 37 % at 20 µg /ml rfBC and 64% at 40µg/ml by THP-1 cells as compared to untreated *M. bovis* BCG (100%).

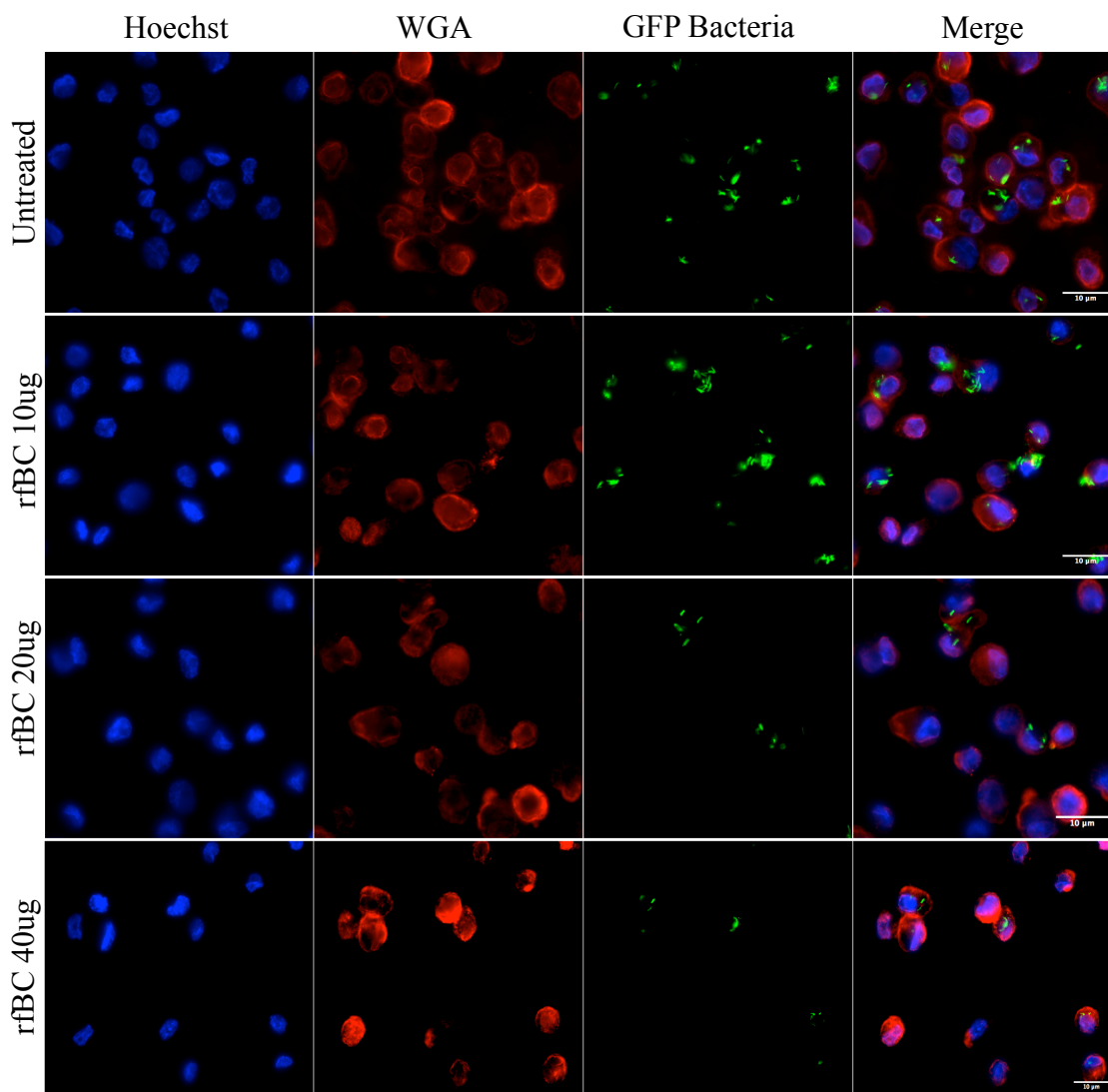
Six hours of treatment with rfBC showed a similar effect as three hours. After six hours, a reduction in *M. bovis* BCG uptake by THP-1 cells was observed at 15% for 10 µg/ml, 33% for 20 µg/ml and 70% for 40-µg/ml rfBC as compared to untreated *M. bovis* BCG (Figure 4.6).



**Figure 4.6:** Effect of recombinant rfBC on the uptake of *M. bovis* BCG by THP-1 cells. Three concentrations (10 µg, 20 µg, 40 µg) of rfBC were used to treat *M. bovis* BCG. rfBC-treated mycobacteria were incubated with THP-1 cells for 3 and 6 hours to study the effect on phagocytosis. *M. bovis* BCG cells released after lysis of THP-1 cells were plated on Middlebrook 7H10 agar and CFUs counted. Each histogram represents the average of 3 experiments. There is a significance difference in uptake between untreated (THP-1 + *M. bovis* BCG – 0 µg/ml) and treated (THP-1 + *M. bovis* BCG + 10, 20 and 40 µg/ml of rfBC). (\* $p < 0.05$ , \*\* $p < 0.01$ ). Assays were performed in triplicate.

#### **4.2.7 Effect of rfBC on uptake of *M. bovis* BCG by THP-1 cell by fluorescence microscopy**

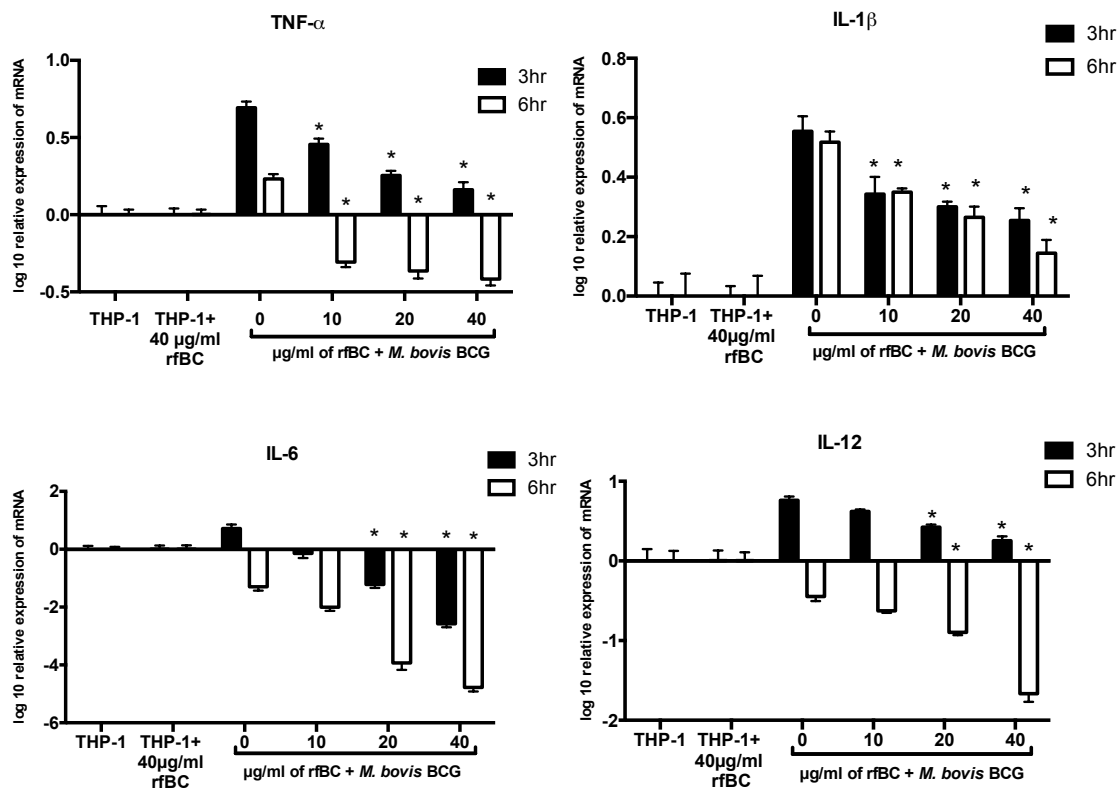
Fluorescence microscopy was performed to observe the effect of rfBC on uptake of *M. bovis* BCG by THP-1 cells after three hours of treatment. Fluorescence microscopy data validated the inhibitory effect of rfBC on the uptake of *M. bovis* BCG by THP-1 cells (Figure 4.7). Thus, rfBC exhibits its uptake inhibitory effect.



**Figure 4.7: Microscopic representation of effect of rfBC on the phagocytosis of *M. bovis* BCG by THP-1 cells.** Fluorescence microscopy was performed to see the uptake of *M. bovis* BCG incubated with different concentration (10, 20 and 40  $\mu\text{g}/\text{ml}$ ) of rfBC by THP-1 cells. *M. bovis* BCG without rfBC was used as a negative control. GFP-expressing *M. bovis* BCG was incubated with rfBC for 1 hour at 37 $^{\circ}\text{C}$  and 1 hour at 4 $^{\circ}\text{C}$  and these rfBC treated bacteria were incubated with THP-1 cells for three hours to examine the effect on phagocytosis. Alexafluor546-conjugated wheat germ agglutinin (WGA) was used to reveal plasma membrane (red), and the nucleus was stained with Hoechst 33342 (Blue), GFP-expressing *M. bovis* BCG (green).

#### **4.2.8 The pro-inflammatory response of THP-1 macrophages during phagocytosis of *M. bovis* BCG treated with and without rfBC.**

Quantitative real-time PCR (qPCR) was used to study the mRNA expression of pro-inflammatory (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12) cytokines (Figure 4.8). In this study we observed that rfBC-treated *M. bovis* BCG shows a down-regulation of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12, at both 3 and 6 h of incubation with THP-1 macrophages. The effect on TNF- $\alpha$  is particularly significant. These levels in cytokine expression were compared to THP-1 only cells with no treatment with rfBC or *M. bovis* BCG.

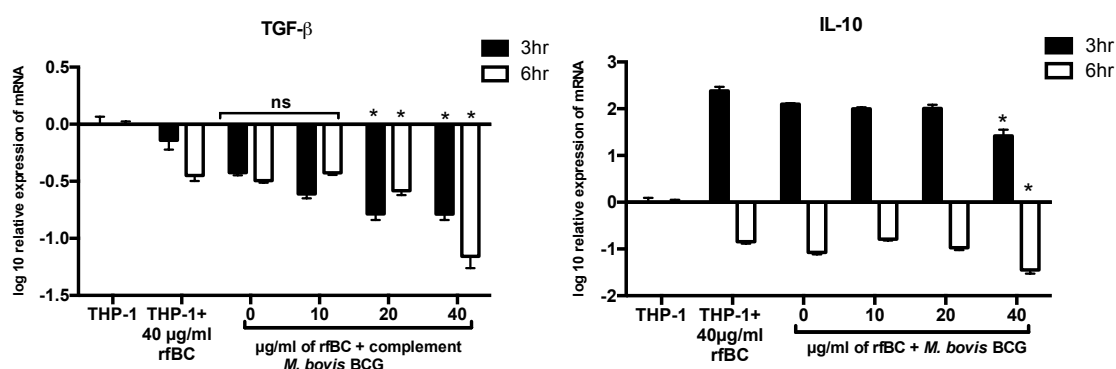


**Figure 4.8: Expression of pro-inflammatory cytokines by THP-1 cells treated with *M. bovis* BCG and different concentrations of rfBC.** qPCR analysis of the expression of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12) at 3 and 6 hours incubation of *M. bovis* BCG and 10  $\mu$ g, 20  $\mu$ g and 40  $\mu$ g of rfBC. The calibrator sample used was THP-1 cells only. The data was normalized to 18S rRNA gene expression which was used as an endogenous control. The data are expressed as a mean of three independent experiments carried out in triplicates (SEM). The log RQ value were plotted to show the gene expression. There is a significance difference established between un-treated samples (THP-1 + *M. bovis* BCG) and treated (THP1 + *M. bovis* BCG + rfBC). The significance was revealed using the 1-way ANOVA test. All comparisons are statistically significant (p  $\leq$  0.05).



#### 4.2.9 The anti-inflammatory response of THP-1 macrophages during phagocytosis of *M. bovis* BCG treated with and without rfBC.

In this study we investigated the anti-inflammatory cytokine TGF- $\beta$  and IL10 gene expressions (Figure 4.9). We found TGF- $\beta$  showed an increase in expression in the presence of rfBC-treated *M. bovis* BCG. The graph shows that TGF- $\beta$  upregulated after 3 hours of the infection in the presence of rfBC and down regulated after 6 hours as compared to untreated *M. bovis* BCG. However, the other anti-inflammatory cytokine, IL-10, was also down-regulated during these initial hours of interaction. THP-1 cells incubated only with rfBC did not show any remarkable changes in cytokine expression.



**Figure 4.9: Expression of anti-inflammatory cytokines by THP-1 cells treated with *M. bovis* BCG and different concentrations of rfBC.** qPCR analysis of the expression of anti-inflammatory cytokines (TGF- $\beta$  and IL-10) at 3 and 6 hours incubation of *M. bovis* BCG and 10  $\mu$ g, 20  $\mu$ g and 40  $\mu$ g of rfBC. The calibrator sample used was THP-1 cells only. The data was normalized to 18S rRNA gene expression which was used as an endogenous control. The data are expressed as a mean of three independent experiments carried out in triplicates (SEM). The log RQ value were plotted to show the gene expression. There is a significance difference established between un-treated samples (THP-1 + *M. bovis* BCG) and treated (THP1 + *M. bovis* BCG + rfBC). The significance was revealed using the 1-way ANOVA test. All comparisons are statistically significant ( $p \leq 0.05$ ).

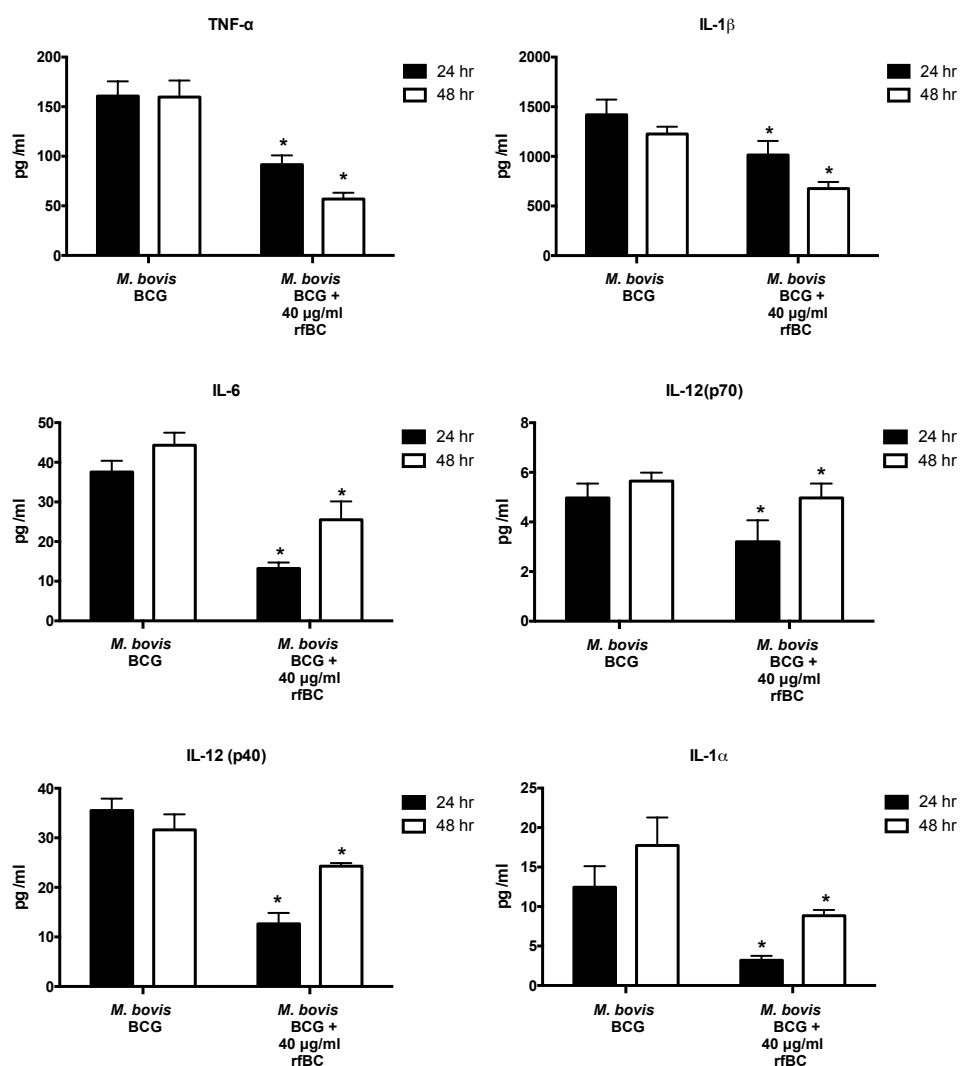
#### **4.2.10 Multiplex Array Analysis to study the cytokines, chemokines and growth factors production by THP-1 cells after phagocytosis *M. bovis* BCG treated with and without rfBC.**

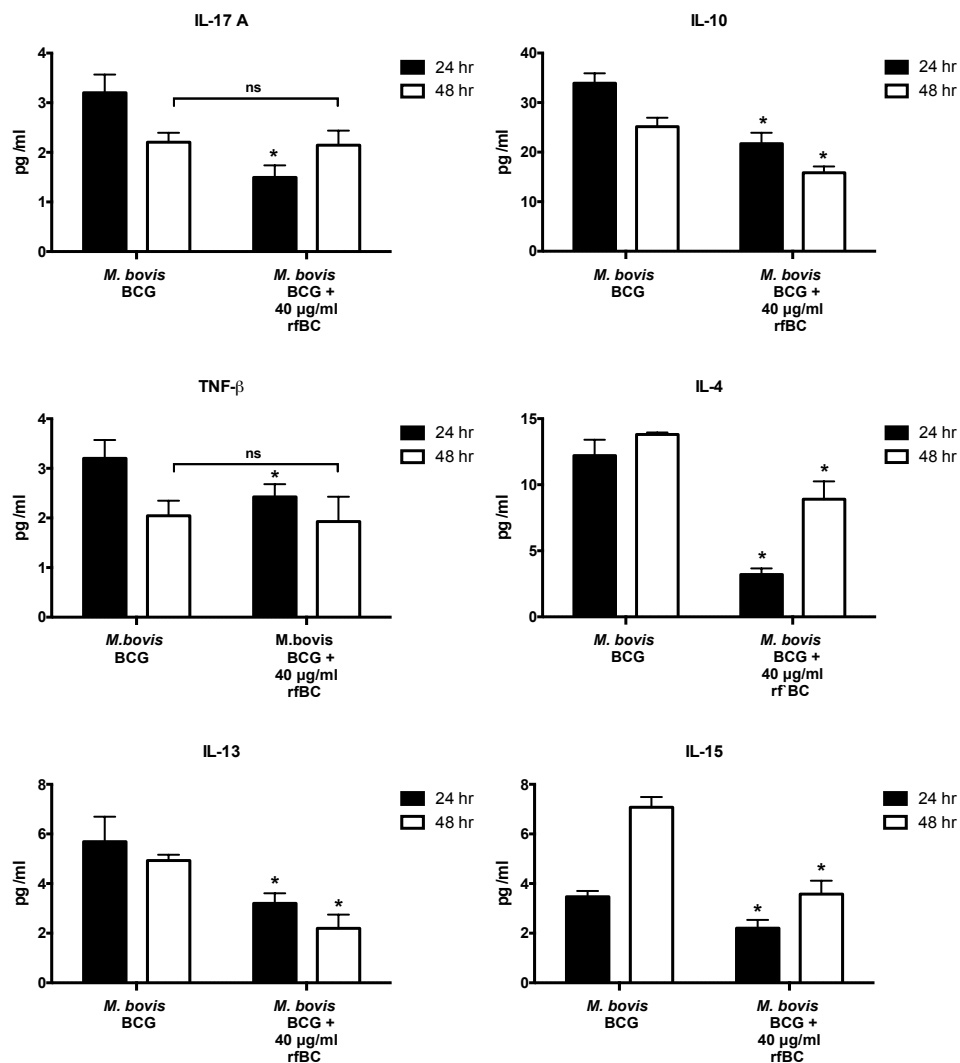
In this study we investigated the immune response after 24h of phagocytosis using multiplex cytokine array analysis to measure the protein levels of cytokines and chemokines produced by THP-1 cells in the supernatant. This study is carried out to investigate the role of conglutinin further in mycobacterial infection, we examined the subsequent immune response by THP-1 macrophages during phagocytosis. We found that rfBC-treated *M. bovis* BCG shown the decrease in the levels of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40, as compared to untreated *M. bovis* BCG (Figure 4.10). A same pattenen of decrease in levels was also observed for the chemokines MCP-3, MDC, Eotaxin, Fractalkine, GRO, growth factors IL-2, FGF-2, G-CSF, GM-CSF, IL-3, IL-7, VEGF and related ligands and receptors IFN- $\gamma$ , FLT-3 and IL-1RA. We observed that *M. bovis* BCG, treated with rfBC, dampened pro-inflammatory response after 24 h of phagocytosis, compared to *M. bovis* BCG only, after a significant inhibition of bacterial uptake by the macrophages. The pro-inflammatory cytokine/chemokines levels at 48 h were similar to levels observed for *M. bovis* BCG only. The dampening of the pro-inflammatory response by conglutinin may also supress the production of important cytokines and chemoattractants (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40) that recuit inflammatory cells and discourage inflammation and tissue remodeling necessary for granuloma formation.

This study also suggested that as rfBC has an anti-inflammatory effect that may suppress anti-mycobacterial immunity and promote the extracellular persistence of the *M. bovis* pathogen in *B. taurus*. A one-way ANOVA was performed to determine significance differences in expression between *M. bovis* BCG (untreated) and *M. bovis* BCG incubated with rfBC of 24 and 48 hours.

#### 4.2.10.1 Multiplex Array Analysis to study the cytokines production by THP-1 cells after phagocytosis *M. bovis* BCG treated with and without rfBC.

Multiplex analysis revealed that THP-1 cells infected with rfBC treated *M. bovis* BCG down regulated the level of cytokines TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40 as compared to THP-1 cells infected with untreated *M. bovis* BCG after 24 and 48 hours. Further suggesting a dampening of pro-inflammatory responses. The decrease in levels of IL-12p40 by rfBC also points to a possible suppression of Th1 cytokines that can be produced by CD4<sup>+</sup> T-cells, leading to a reduction in IFN- $\gamma$  which is also critical for sustained intracellular killing by macrophages and granuloma formation. The decrease of IL-10 by rfBC also suggests the suppression of anti-mycobacterial responses by the macrophage

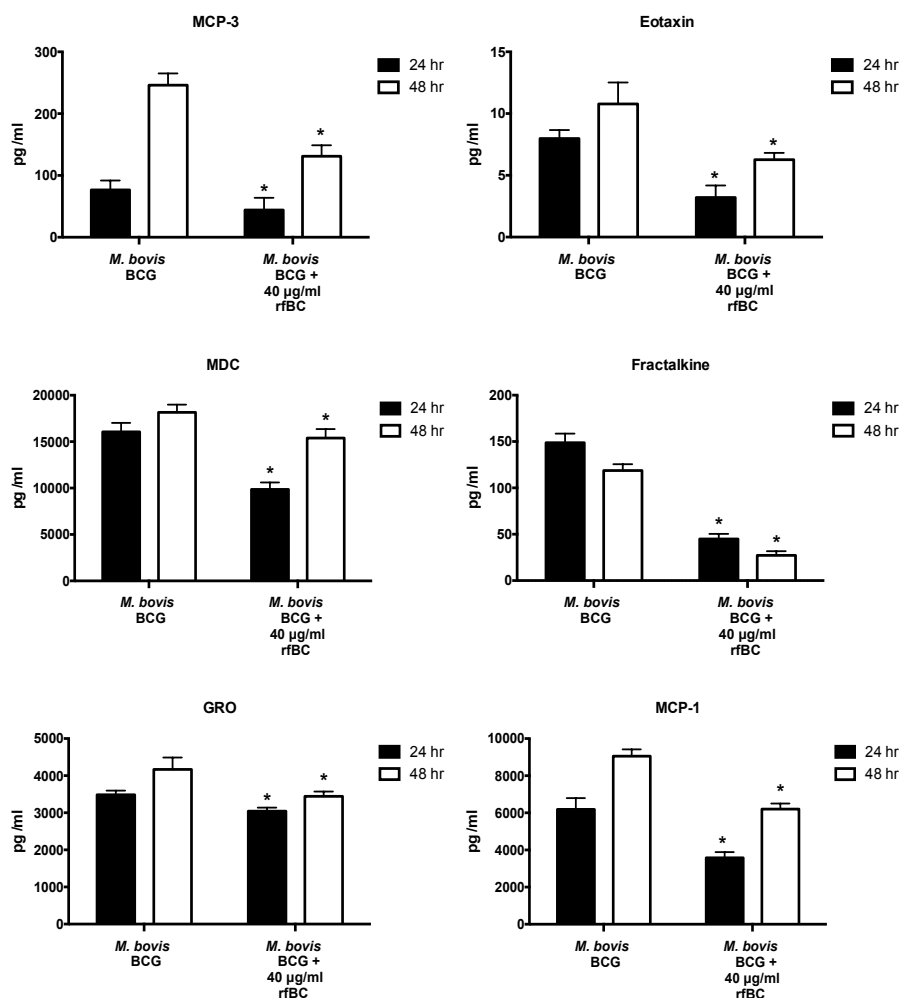




**Figure 4.10: Multiplex cytokine analysis of supernatants collected at 24 h after phagocytosis of *M. bovis* BCG by THP-1 macrophages treated with rfBC.** Supernatant from late time points (24 and 48 h) (X-axis) were used for the measurement of the levels of cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12 (p40,p70), IL-1 $\alpha$ , IL-17, TNF- $\beta$ , IL-10, IL-4, IL-13 and IL-15 ) inside the THP-1 by MagPix Milliplex kit (EMD Millipore). Assays were performed in duplicate Bars represents  $\pm$  2 standard deviations. A one-way ANOVA was performed to determine significance differences in expression between *M. bovis* BCG (untreated) and *M. bovis* BCG incubated with rfBC of 24 and 48 hours. All these comparisons were significant ( $P < 0.05$ ), except where shown [not significant (ns),  $P > 0.05$ ].

#### 4.2.10.2 Multiplex Array Analysis to study the chemokines production by THP-1 cells after phagocytosis *M. bovis* BCG treated with and without rfBC.

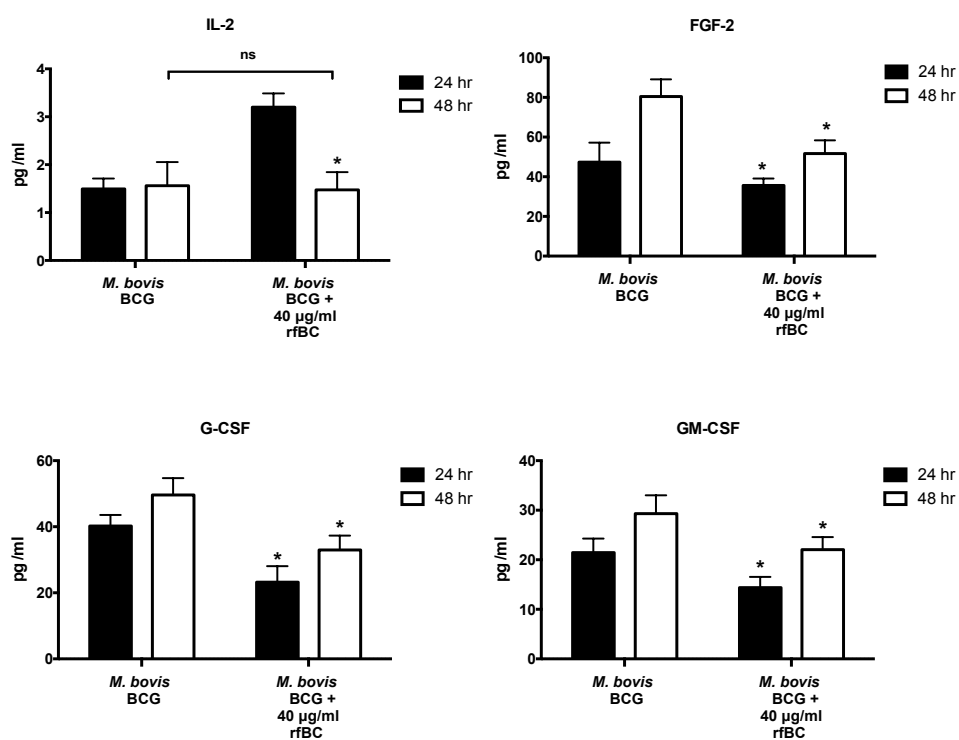
Multiplex analysis revealed that THP-1 cells infected with rfBC treated *M. bovis* BCG down regulated the level of chemokines MCP-3, MDC, Eotaxin, Fractalkine and GRO as compared to THP-1 cells infected with untreated *M. bovis* BCG after 24 and 48 hours.

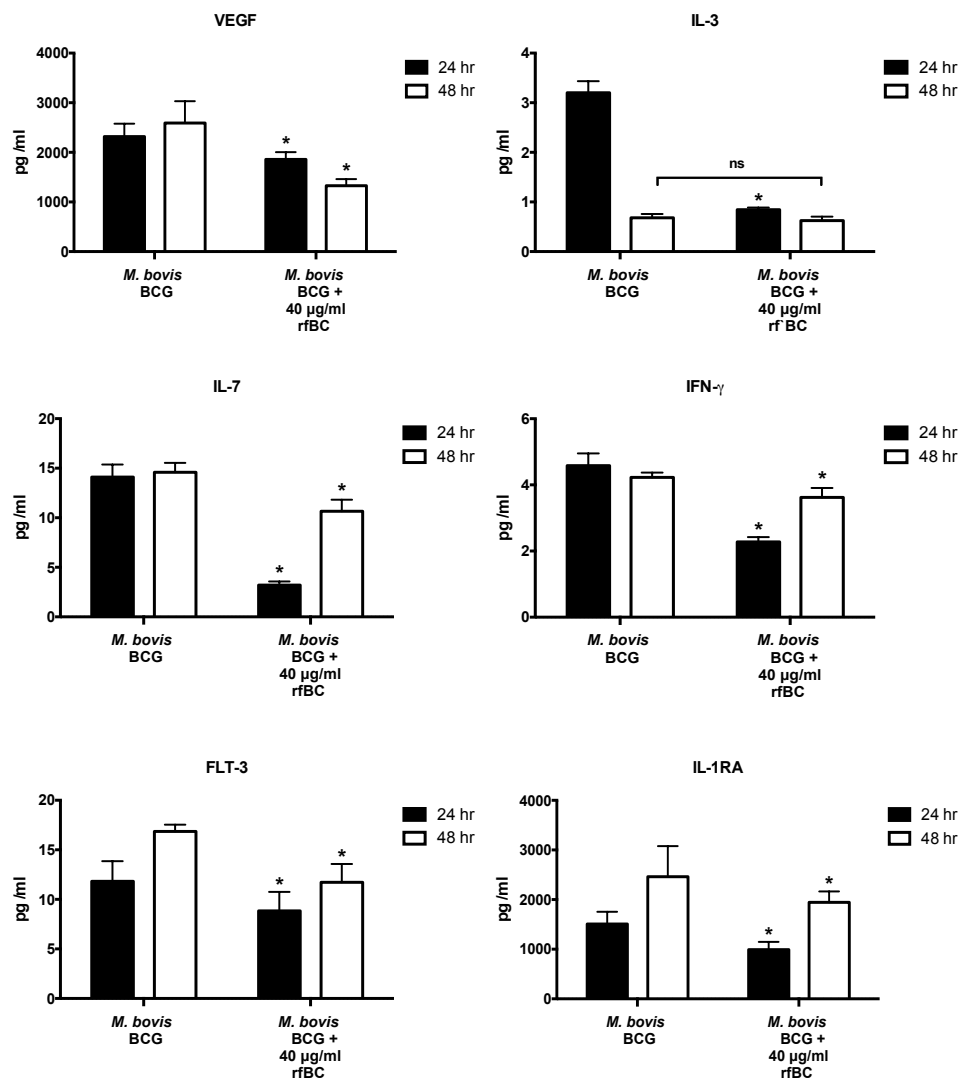


**Figure 4.11: Multiplex chemokines analysis of supernatants collected at 24 h after phagocytosis of *M. bovis* BCG by THP-1 macrophages treated with rfBC.** Supernatant from late time points (24 and 48 h) (*X*-axis) were used for the measurement of the levels of cytokines (MCP-3, MDC, Eotaxin, Fractalkine, GRO and MCP-1) inside the THP-1 by MagPix Milliplex kit (EMD Millipore). Assays were performed in duplicate. Bars represents  $\pm 2$  standard deviations. A one-way ANOVA was performed to determine significance differences in expression between *M. bovis* BCG (untreated) and *M. bovis* BCG incubated with rfBC of 24 and 48 hours. All these comparisons were significant ( $P < 0.05$ ).

#### 4.2.10.3 Multiplex Array Analysis to study the growth factors and related ligands and receptors production by THP-1 cells after phagocytosis *M. bovis* BCG treated with and without rfBC.

The supernatants were collected from phagocytosis assay of *M. bovis* BCG in the presence or absence of rfBC at 24 h and 48 h time point. In this study multiplex analysis shown that THP-1 cells infected with *M. bovis* BCG incubated with rfBC decreased the level of growth factors IL-2, FGF-2, G-CSF, GM-CSF, IL-3, IL-7, VEGF and related ligands and receptors IFN- $\gamma$ , FLT-3 and IL-1RA as compared to THP-1 cells infected with untreated *M. bovis* BCG after 24 and 48 hours.



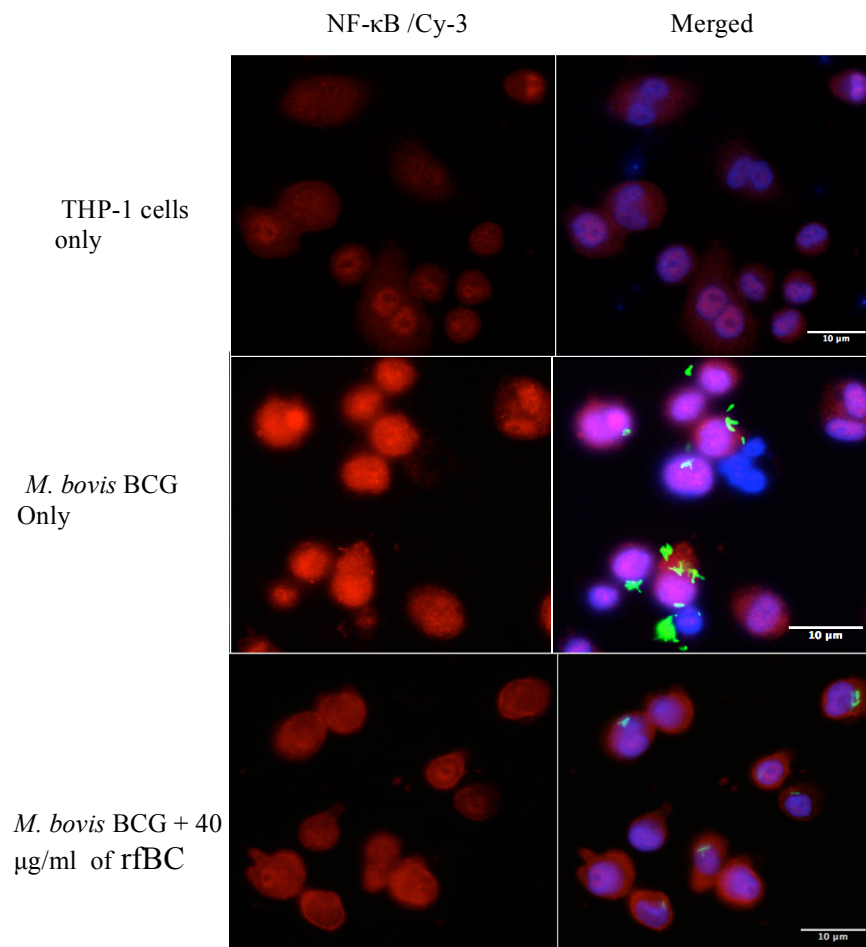


**Figure 4.12: Multiplex analysis of growth factors and related ligands and receptors supernatants collected at 24 and 48h after phagocytosis of *M. bovis* BCG by THP-1 macrophages treated with rfBC.** Supernatant from late time points (24 and 48 h) (X-axis) were used for the measurement of the levels of growth factors and related ligands and receptors production. We studied the measurement of the levels of growth factors (IL-2, FGF-2, G-CSF, GM-CSF, VEGF, IL-3 and IL-7) and related ligands and receptors (IFN- $\gamma$ , FLT-3 and IL-1RA) inside the THP-1 by MagPix Milliplex kit (EMD Millipore). Assays were performed in duplicate. Bars represents  $\pm$  2 standard deviations. A one-way ANOVA was performed to determine significance differences in expression between *M. bovis* BCG (untreated) and *M. bovis* BCG incubated with rfBC of 24 and 48 hours. All these comparisons were significant ( $P < 0.05$ ), except where shown [not significant (ns),  $P > 0.05$ ].

#### **4.2.11 NF- $\kappa$ B cytoplasm to nucleus translocation during phagocytosis of *M. bovis* BCG (with and without rfBC) by THP-1 cells.**

To investigate the inflammatory response further, an experiment was conducted to visualise the translocation of NF- $\kappa$ B in THP-1 cells infected with *M. bovis* BCG, with and without rfBC. NF- $\kappa$ B, is a key transcription factor that modulates the expression of a number of pro-inflammatory cytokines. We studied the translocation of NF- $\kappa$ B in THP-1 cells stained with an antibody against the p65 subunit of NF- $\kappa$ B performed analysis using fluorescence microscopy. We observed that NF- $\kappa$ B translocation was more pronounced in untreated *M. bovis* BCG as compared to THP-1 only. However, virtually no translocation was observed for rfBC-treated *M. bovis* BCG, thus suggesting the downregulation of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) and chemokines (MCP-1) (Figures 4.10, 4.11).





**Figure 4.13: NF- $\kappa$ B translocation in THP-1 cells after uptake of rfBC bound *M. bovis* BCG.** Fluorescence microscopy was performed to investigate the cytoplasm to nucleus translocation of NF- $\kappa$ B in THP-1 cells infected with GFP-*M. bovis* BCG with or without 40  $\mu$ g/ml of rfBC. These were compared with GFP-*M. bovis* BCG only and THP-1 cells only were used as a negative control. Bacteria and THP-1 cells were incubated for 3 hours for phagocytosis to occur. Cells were washed, fixed, permeabilised and incubated with rabbit anti-NF- $\kappa$ B p65 antibodies, followed by Cy3-conjugated goat anti-rabbit antibody (red). The nucleus was stained with DAPI (blue).

### 4.3 Discussion

In the present study, we have studied the bovine collectin conglutinin and its role in tuberculosis host-pathogen interactions. *M. bovis* BCG (Bacillus Calmette-Guerin) was used as a model for *M. bovis*, to study the interaction of rfBC with mycobacteria. THP-1 (human monocyte cell line) was used as a model for the macrophages. We have demonstrate that a homotrimeric recombinant truncated form of rfBC, composed of the  $\alpha$ -helical neck region and the CRD (Wang *et al.*, 1995), was able to bind to *M. bovis* BCG and has a major influence on its phagocytosis by THP-1 macrophages. rfBC significantly inhibits the uptake of *M. bovis* BCG by THP-1 cells and this occurs in a dose dependent manner. These observations suggest that rfBC is masking mycobacterial ligands and inhibiting uptake, possibly through mannose receptors.

Collectins can bind to the carbohydrate ligands on the surface of microorganisms, inducing aggregation and stimulating phagocytosis through specific receptors on phagocytes (Holmskov, 2000). There are various receptors such as C1qR/CD93, calreticulin, and glycoprotein-340 present on the outer surface of immune cells, those interacts with the collectins and helps phagocytes in the phagocytosis, killing and elimination of microorganisms from infected host (Tenner, 1993; Malhotra *et al.*, 1990; Holmskov *et al.*, 1997).

It has previously been shown that conglutinin-neck-CRD region is capable of binding to lipopolysaccharides from Gram-negative bacteria (Wang *et al.*, 1995). It has been reported that conglutinin shows affinity for mannan present on the surface of microorganism such as zymosan (Dec *et al.*, 2012). Zymosan (yeast cell wall component) is a protein and sugar molecule in which sugar is composed of many subunits of mannose and shows strong binding to rfBC (Dec *et al.*, 2012). Collectins such as SP-D have been shown to bind to the lipoarabinomannan (LAM), important cell wall component of *M. tuberculosis* and *M. bovis* (Ferguson, 2002). In the previous chapter, we observed the rfBC binding with *M. smegmatis*, a non-pathogenic species of mycobacteria, but in this chapter, we investigated the rfBC binding with more specific and relevant strain of mycobacteria *M. bovis* BCG. The binding of rfBC with *M. bovis* BCG has not been previously investigated. In this chapter, we performed binding assays using different concentration of rfBC to *M. bovis* BCG and it was observed that rfBC in the presence of calcium was able to strongly bind to *M. bovis* BCG (Figure

4.1). Our result showed 3 to 4 folds less binding between rfBC and *M. bovis* BCG in the existence of EDTA and more strong binding observed in the presence of calcium, that confirms previous finding that rfBC is a calcium dependant lectin (Wang *et al.*, 1995). A concentration of 20 µg/ml of rfBC was enough to reach saturation of binding. This is also the first time rfBC has been shown to bind with Gram-positive bacteria. Immunofluorescence microscopy was also performed to visually confirm the binding of rfBC to *M. bovis* BCG (Figure 4.2).

In this chapter, the direct effect of rfBC on the growth of *M. bovis* BCG was studied. The results showed that rfBC was able to inhibit the growth of *M. bovis* BCG in a dose dependent manner. There is 25% reduction in growth observed for 10 µg/ml, 44% for 20 µg/ml and 90% for 40 µg/ml of rfBC. The inhibition results are statistically significant at all concentration observed (Figure 4.3). This direct inhibition role of rfBC on mycobacteria has not been studied before. It has previously been reported that conglutinin played a role in the survival of mice infected with pathogenic strains of *Salmonella typhimurium*, *Pasteurella septica*, *Klebsiella pneumoniae*, *Listeria monocytogenes* and *Streptococcus pyogenes* (Friis-Christiansen, 1990). Friis-Christiansen in 1990 also confirmed the study, that mice injected with conglutinin prolonged the survival of infected mice with *S. typhimurium*. They suggested that conglutinin serve as a mediator between the bacteria and the immune cells (Friis-Christiansen 1990).

SP-D has been shown to inhibit the growth of *E. coli*, *Legionella pneumophila*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* by increasing the cell membrane permeability (Wu *et al.*, 2003), but not in the case of mycobacteria (Ariki, 2011). As we observed that rfBC have a direct inhibitory effect on the *in vitro* growth of *M. bovis* BCG (Figure 4.3). We performed experiments to find out whether rfBC increased the cell membrane permeability of the *M. bovis* BCG and accounting for growth inhibition. We found that direct effect was bacteriostatic in nature, rather than bactericidal, as virtually no bacterial cell lysis was observed on rfBC-treated *M. bovis* BCG, as determined by propidium iodide (PI) staining (Figures 4.4). This study show for the first time that rfBC has a bacteriostatic effect, which could have important finding in the control of mycobacterial infection in cattle.

It has been reported that collectins binds directly with the carbohydrates present on the microbial surface causing bacterial aggregation and virus neutralization and assists phagocytic cells in phagocytosis, act as an opsonin (Hansen and Holmskov, 1998). SP-A has been shown to enhance the uptake of *M. tuberculosis* by phagocytic cells such as macrophages and this is due to the up-regulation of macrophage-mannose receptor (Gaynor *et al.*, 1995). In previous studies, SP-D has shown different effects on phagocytosis of pathogens. *M. tb* treated with SP-D showed the reduction in the uptake by macrophages (Ferguson *et al.*, 1999). SP-D CRD region binds to the cell wall components of *M. tuberculosis* such as mannosyl units (mannose caps) of lipoglycan and lipoarabinomannan (LAM) (Ferguson *et al.*, 1999).

The effect of rfBC on the uptake of *M. bovis* BCG or any other mycobacterium species by macrophages has not investigated before. In this chapter, we studied the effect of rfBC on the uptake of *M. bovis* BCG by THP-1 cells. For this purpose *M. bovis* BCG incubated with different concentration 10 µg, 20 µg and 40 µg/ml of rfBC for 2 hours to treat with THP-1 cells. We found rfBC significantly inhibit the uptake of *M. bovis* BCG by macrophages (THP-1 cells). rfBC-treated *M. bovis* BCG showed reduced uptake by THP-1 cells, compared to untreated *M. bovis* BCG, with up to 63% (40 µg/ml of rfBC), 35% (20 µg/ml of rfBC) and 18% (10 µg/ml of rfBC) inhibition (Figure 4.6). The reasons for this significant inhibition in uptake of *M. bovis* BCG by THP-1 cells is possibly rfBC binds with surface antigens of *M. bovis* BCG such as mannose receptors on macrophages. Previously it has been reported that surfactant protein SP-D strongly binds to lipoglycan lipoarabinomannan (LAM) of virulent *M. tb*, strains. The complex of the *M. tuberculosis*-SP-D inhibited the binding of mycobacteria to the human macrophages (Ferguson *et al.*, 2002). Similar to SP-D, we found rfBC to be able to significantly inhibit the uptake of mycobacteria by macrophages (THP-1 cells). One of the main route for *M. tuberculosis* phagocytosis by macrophages is via the mannose receptor (Schlesinger, 1993). It has been studied that conglutinin shows haemagglutination inhibition and neutralizing effect against influenza A virus and rota viruses (Hartshorn *et al.*, 1993; Reading *et al.*, 1998).

Collectins such as SP-A and SP-D have the ability to enhance or inhibit the inflammatory responses against the pathogens (Kuroki *et al.*, 2007). In this chapter, we investigated the effect of rfBC on the inhibition of mycobacterial growth in to the

THP-1 cells by engagement of pro-inflammatory and anti-inflammatory signaling processes. Pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12 production was down regulated at 3 and 6 hours, suggesting the rfBC role in the adaptive immunity. Three and six hour responses of rfBC were assessed by qPCR, later investigated the cytokines, chemokine and growth factors levels at 24 and 48 hours after rfBC-*M. bovis* BCG interaction with THP-1 cells by performing multiplex array analysis. Multiplex analysis showed down regulation of cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-12p40, IL-12p70, IL-17A and TNF- $\beta$ ) (Figure 4.10) for rfBC treated *M. bovis* BCG. It has been observed that mostly chemokines (MCP-3, MDC, Eotaxin, Fractalkine, GRO and MCP-1) (Figure 4.11) and growth factors proteins (IL-2, FGF-2, GM-CSF, VEGF, IFN- $\gamma$ , FLT-3 and IL-1RA) (Figure 4.12) were down regulated for rfBC bound *M. bovis* BCG. TNF- $\alpha$ , IL-1 $\beta$  and IL-12 gene expression in THP-1 cells infected with rfBC treated *M. bovis* BCG was reduced in early responses (3 and 6 hours) as well as late responses (24 and 48 hours) as compared to untreated *M. bovis* BCG (Figure 4.8). TNF- $\alpha$  also plays a major contribution in the formation of granuloma in tuberculosis infection. TNF- $\alpha$  mediates the recruitment of immune cells in the granuloma (Mohan *et al.*, 2001).

The mRNA level of IL-1 $\beta$  was down regulated in THP-1 cells treated with *M. bovis* BCG bound with rfBC after 3 and 6 hours of infection (Figure 4.8). Multiplex analysis 24 and 48 hours post infection revealed the same result of down regulation of IL-1 $\beta$  response was consistent with early response. In bovine tuberculosis, IL-1 $\beta$  is produced by monocytes, macrophages and dendritic cells. It has been studied that IL-1 $\beta$  receptor deficient mice are more likely infected by *M. tb* and also lack the ability to form granuloma (Juffermans *et al.*, 2000).

NF- $\kappa$ B is an important transcription factor that regulates the synthesis of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ . We studied the NF- $\kappa$ B translocation in THP-1 cells stained with an antibody against the NF- $\kappa$ B and analysed by fluorescence microscope. We observed no nuclear translocation of NF- $\kappa$ B in response to rfBC bound *M. bovis* BCG as compared to untreated *M. bovis* BCG (Figure 4.13). This result is further evidence of a dampening of the inflammatory response in the presence of rfBC and also suggested the down regulation of pro-inflammatory cytokines and chemokines.

IL-6 assist *M. avium* multiplication and survival in vitro by inhibiting TNF- $\alpha$  and IL-1 $\beta$  secretion (Schindler *et al.*, 1990). In this study, IL-6 mRNA expression was down regulated in the THP-1 cells treated with *M. bovis* BCG bound with rfBC after 3, 6, 24 and 48 hours of the infection (Figure 4.8, Figure 4.10).

The expression of IL-12 was up regulated in THP-1 cells incubated with *M. bovis* BCG bound with rfBC as compared to untreated *M. bovis* BCG in 3 hours of infection and observed down regulation in 6 hours (Figure 4.8). This result suggested that 20 $\mu$ g and 40 $\mu$ g/ml rfBC increases the expression of IL-12 in the early stage of infection but down regulated after 6 hours of infection. IL-12 also involved in the activation of antigen-specific lymphocytes, which form granuloma (Cooper *et al.*, 1997). IL-12p40 and IL-12p70 were down regulated after 24 and 48 hours of the infections.

Our result shows that TGF- $\beta$  was upregulated after 3 hours of the infection in the presence of rfBC and down regulated after 6 hours as compared to untreated mycobacteria (Figure 4.9). TGF- $\beta$  involved in the down regulation of pro-inflammatory cytokines release such as TNF- $\alpha$  and IL-1 $\beta$  (Ruscetti *et al.*, 1993). It is synthesised by macrophages and dendritic cells in response to tuberculosis infection. TGF- $\beta$  helps in the mycobacterial survival by preventing the formation of reactive oxygen and nitrogen radicals in the macrophages (Ding *et al.*, 1990).

IL-10 helps in the survival of intracellular mycobacteria by deactivating macrophages to produce IL-12 and TNF- $\alpha$ , which decreases the INF- $\gamma$  synthesis by CD4<sup>+</sup>. Less expression of INF- $\gamma$  leads to decrease formation of reactive nitrogen and oxygen intermediates, which helps in the survival of mycobacteria (Gazzinelli *et al.*, 1992). It has been shown in the mice that IL-10 stops CD4<sup>+</sup> cell responses, which inhibit phagosome maturation and also stops mycobacterial antigen presentation to the immune cells (Bobadilla *et al.*, 2013). In this study, the mRNA level of IL-10 in THP-1 cells was down regulated at 3 and 6 hours (Figure 4.9) and also consistent results we observed after 24 and 48 hours (Figure 4.10) of infection with *M. bovis* BCG bound with c rfBC as compared to *M. bovis* BCG only.

It has been studied that IL-4 is not only produced by CD4<sup>+</sup> their primary source of production but also produced by CD8<sup>+</sup> T-cells in pulmonary tuberculosis (Van *et al.*, 2000). Over production of IL-4 in mice promotes infection progress and reactivate

latent tuberculosis infection (Hernandez *et al.*, 1996). In this study, multiplex data revealed the down regulation of IL-4 after 24 and 48 hours of infection in the presence of rfBC as compared to untreated mycobacteria (Figure 4.10).

In this study we observed that rfBC treated *M. bovis* BCG down regulated the level of chemokines (MCP-3, MDC, Eotaxin, Fractalkine, GRO and MCP-1) expression in infected THP-1 cells after 24 and 48 hours as compared to untreated *M. bovis* BCG. It has also been shown that monocyte chemo-attractant protein-1 (MCP-1) increase Th2 polarization and recruits immune cells (monocytes, memory T-cells, dendritic cells) to the site of infection (Rose *et al.*, 2003). Monocyte chemo-attractant protein-3 (MCP-3) is secreted by macrophages and reported higher level in bronchoalveolar fluid of tuberculosis patient (Ruhwald *et al.*, 2009). Growth-related oncogene-1 (GRO1) is also produced by macrophages, neutrophils and epithelial cells. GRO assists in the recruitment of neutrophils and monocytes to the infectious site (Nakagawa *et al.*, 1994). MDC (macrophage derived chemokine) is one of the important chemokines helps in the attraction of monocytes, dendritic cells and IL-2 activated natural killer cells to the site of infection (Godiska *et al.*, 1997).

We also investigated the level of growth factors and related ligands and receptors by THP-1 cells treated with *M. bovis* BCG bound with rfBC as compared to THP-1 cells infected with *M. bovis* BCG only after 24 and 48 hours. In this study multiplex analysis showed decreased level of growth factors (IL-2, FGF-2, G-CSF, GM-CSF and VEGF) and related ligands and receptors (IFN- $\gamma$ , FLT-3 and IL-1RA) (Figure 4.12) by THP-1 cells treated with *M. bovis* BCG bound with rfBC as compared to THP-1 cells treated with *M. bovis* BCG only after 24 and 48 hours. Granulocytes-colony stimulating factor (G-CSF) is one of the important growth factor secreted by monocytes, fibroblasts and endothelial cells (Schneider *et al.*, 2005). G-CSF encourages the proliferation and maturation of neutrophil from the precursor (Demetri and Griffin, 1991).

The down regulation of the pro-inflammatory response by rfBC may also inhibit the production of important cytokines and chemoattractants such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40, IFN- $\gamma$  those recruit inflammatory cells and prevent inflammation and granuloma formation (Feng *et al.*, 2006; Smith *et al.*, 1997). The down regulation of IL-12p40 by rfBC also a possible cause of suppression of the Th1 cells that results in

the reduction in the IFN- $\gamma$  production, which is also an important component for intracellular killing by macrophages and helps in granuloma formation. In this study we also observed the down regulation of growth factors VEGF, GM-CSF and GRO in the THP-1 cells infected by rfBC bound *M. bovis* BCG as compared to untreated *M. bovis* BCG, further confirming the dampening of inflammatory responses. In vivo, macrophages phagocytose mycobacteria after entry in to the host but ultimately fail to destroy them and provide hostile environment for multiplication. rfBC inhibition of phagocytosis of these bacteria in to macrophages may be protective by keeping them extracellular where these can be easily cleared by the host immune response.



**Chapter 5 – Investigating the effect of complement on the interactions of rfBC and *M. bovis* BCG and its implications for mycobacterial infection.**

## 5.1 Introduction

The complement system provides primary protection against pathogens. Pathogenesis by microorganisms depends on their ability to evade killing by host immune system. It is known that mycobacteria are highly adapted to living inside macrophages. To facilitate this, mycobacteria can activate complement, which can promote uptake into phagocytes via complement receptors.

In this chapter, the binding of the bovine conglutinin fragment (rfBC) with *M. bovis* BCG with and without complement deposition has been studied, to examine the effect on phagocytosis by THP-1 cells and the subsequent immune response. The first aim in this study was to examine the complement consumption by *M. bovis* BCG. It has been previously shown, that the activation of complement system predominantly occurs via alternative pathway (Carroll *et al.*, 2009). In this study, we show that the interaction between *Mycobacterium bovis* BCG and complement system in normal human serum was sufficient to activate the complement cascade, culminating in an average of 90% of complement consumption with C3b being deposited on the bacterial surface. This confirms that *M. bovis* BCG can activate complement.

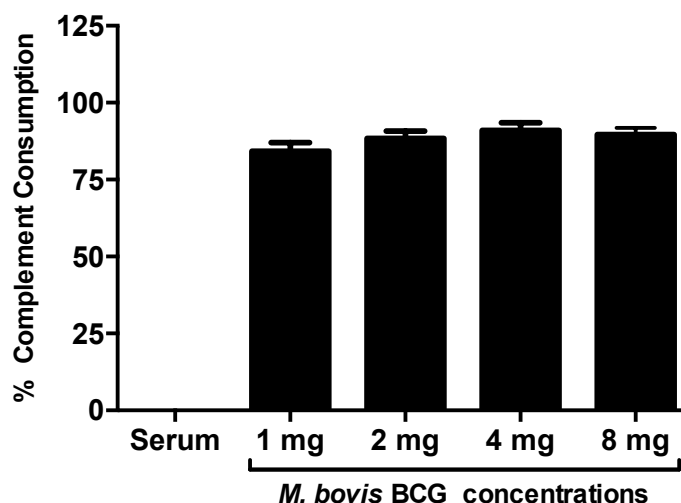
The second aim in this study was to assess the complement dependent interactions of rfBC with THP-1 cells on phagocytosis. We observed that rfBC inhibits phagocytosis of *M. bovis* BCG deposited with complement proteins by THP-1 cells. Conglutinin is a known binder of iC3b, there is a possibility of conglutinin interfere with complement receptor mediated uptake via CR3 and CR4 receptors by masking interaction with iC3b deposited on the mycobacterial surface.

The third aim in this study was to investigate the cytokine expression of THP-1 cells during *M. bovis* BCG infection in the presence of rfBC and/or complement. We also observed that rfBC modulated the downstream inflammatory response, which is important for activating the adaptive immune response encouraging containment of mycobacterial infection.

## 5.2 Results

### 5.2.1 Complement consumption assay with *M. bovis* BCG

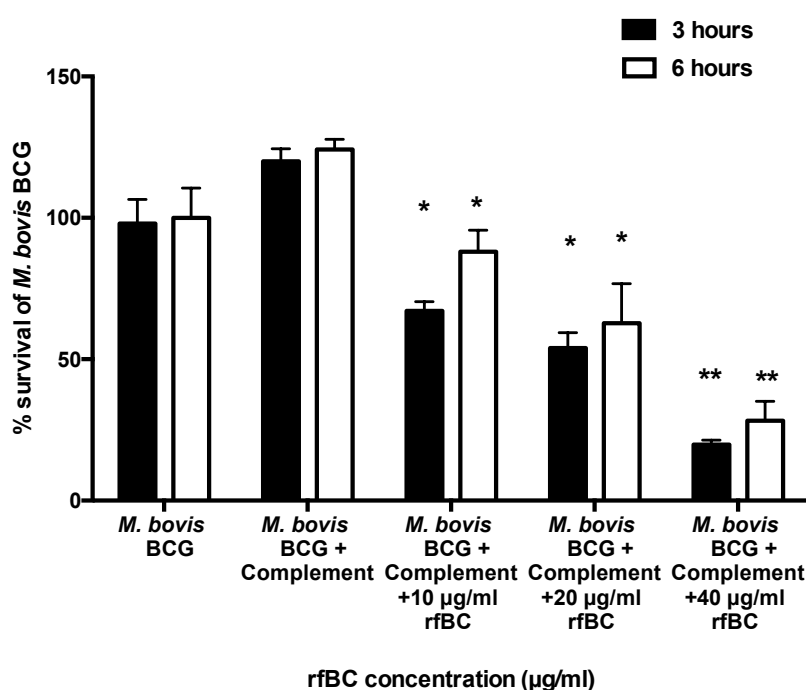
The activation of the complement system in human serum by the alternative complement pathway by *M. bovis* BCG is shown in Figure 5.1. At different doses of *M. bovis* BCG consumed nearly 90% of complement which was activated by alternative pathway. To optimised and lysis in complement deposited samples were compared with serum only. Different amount (1mg, 2 mg, 4 mg and 8 mg) of *M. bovis* BCG were used to identify which one would present the best consumption. Figure 5.1 shows similar consumption for all conditions that is around 90% consumption.



**Figure 5.1: Activation of alternative pathway by *M. bovis* BCG in normal human serum.** Different concentrations (1mg, 2 mg, 4 mg and 8 mg) of *M. bovis* BCG from fresh culture  $OD_{600nm} = 1$  was taken. An *M. bovis* BCG bacterial pellet 10 $\mu$ l packed volume corresponding to 1 mg dry weight (Carrol *et al.*, 2009). Percentage of complement consumption was plotted with serum as a control for no consumption. Results are expressed as the mean of duplicates from one experiment  $\pm$  S.D.

### 5.2.2 Effect of rfBC on the phagocytosis of complement deposited *M. bovis* BCG by THP-1 cells

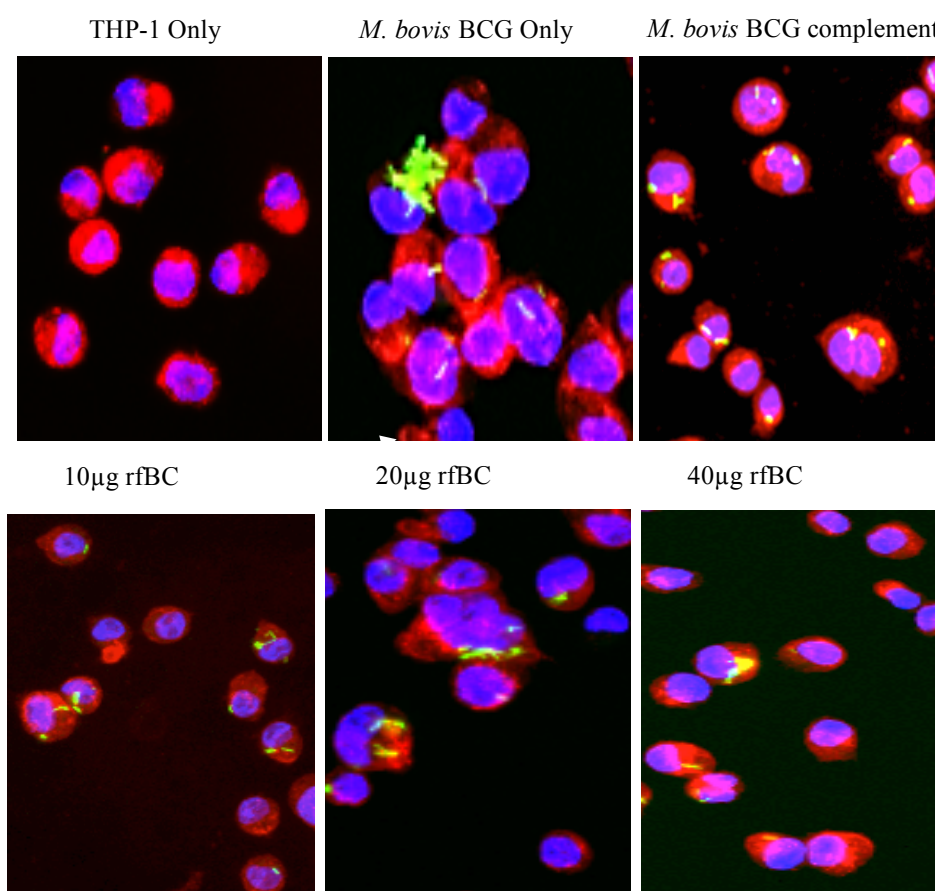
In this study, *M. bovis* BCG were deposited by complement proteins by activating alternative the pathway. Complement deposited *M. bovis* BCG was treated with three concentration (10 $\mu$ g, 20 $\mu$ g, 40 $\mu$ g) of rfBC in the presence of 5mM calcium chloride for 3 and 6 hours (Figure 5.2). The results show that rfBC decreased complement-deposited mycobacterial uptake by THP-1 cells at both time points. Three independent experiments were carried out and all showed the same trend. The results are statistically significant.



**Figure 5.2: Effect of rfBC on the phagocytosis of complement coated *M. bovis* BCG by THP-1 cells.**  $1 \times 10^{-2}$  dilution of *M. bovis* BCG after lysis of THP-1 cells were plated on middle brook 7H10 agar plates. There is a significance difference established between un-treated samples (*M. bovis* BCG + complement) and (*M. bovis* BCG + complement + rfBC) (\* $p < 0.05$ , \*\* $p < 0.01$ ). Each histogram represents the average of 3 experiments.

### 5.2.3 The effect of rfBC on the uptake of complement deposited *M. bovis* BCG by THP-1 cells after 3 hours by fluorescence microscopy

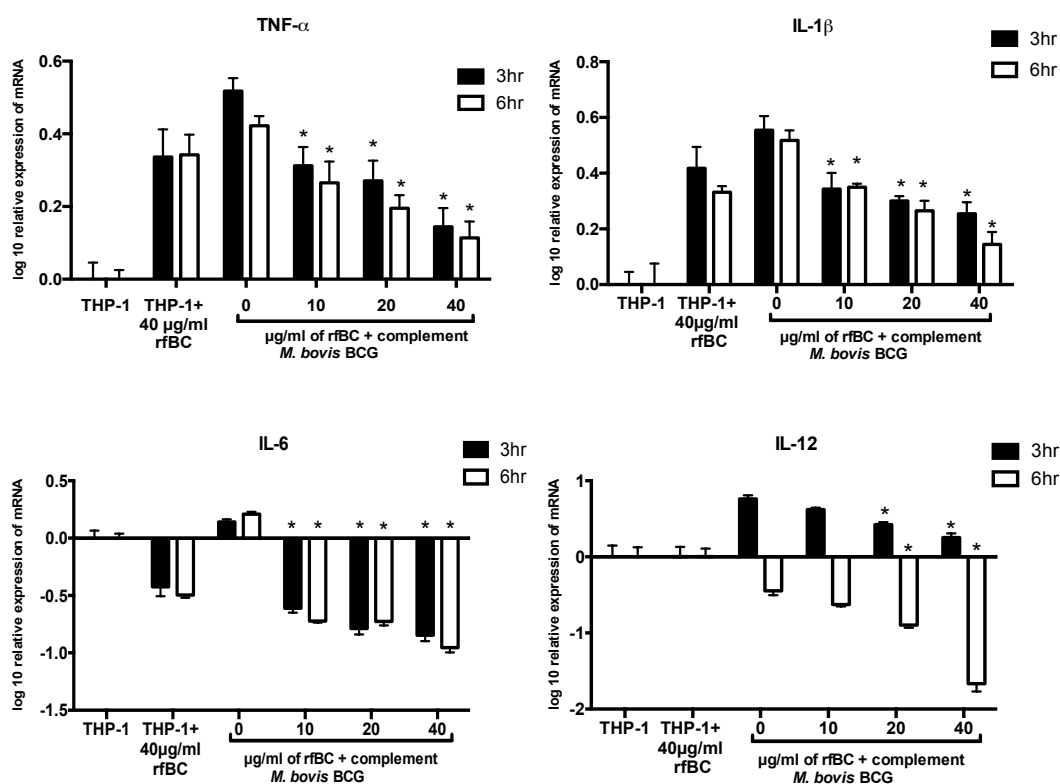
In this study, fluorescence microscopy was performed to observe the effect of rfBC on uptake of complement deposited *M. bovis* BCG by THP-1 cells after three hours of phagocytosis. Fluorescence microscopy data validated the inhibitory effect of rfBC on the uptake of *M. bovis* BCG by THP-1 cells (Figure 5.2). Thus, rfBC exhibits its uptake inhibitory effect (Figure 5.3).



**Figure 5.3: Fluorescence microscopy showing the effect of rfBC on the phagocytosis of complement deposited *M. bovis* BCG by THP-1 cells.** Fluorescence microscopy was performed to see the uptake of complement coated *M. bovis* BCG incubated with different concentration (10,20 and 40 µg/ml) of rfBC in to the THP-1 cells. *M. bovis* without protein used as a negative control. *M. bovis* BCG coated with complement was used as a positive control. *M. bovis* BCG coated with complement and rfBC treated with THP-1 cells for three hours to examine the phagocytosis impact.

### 5.2.4 The pro-inflammatory response of THP-1 macrophages during phagocytosis of complement deposited *M. bovis* BCG treated with and without rfBC.

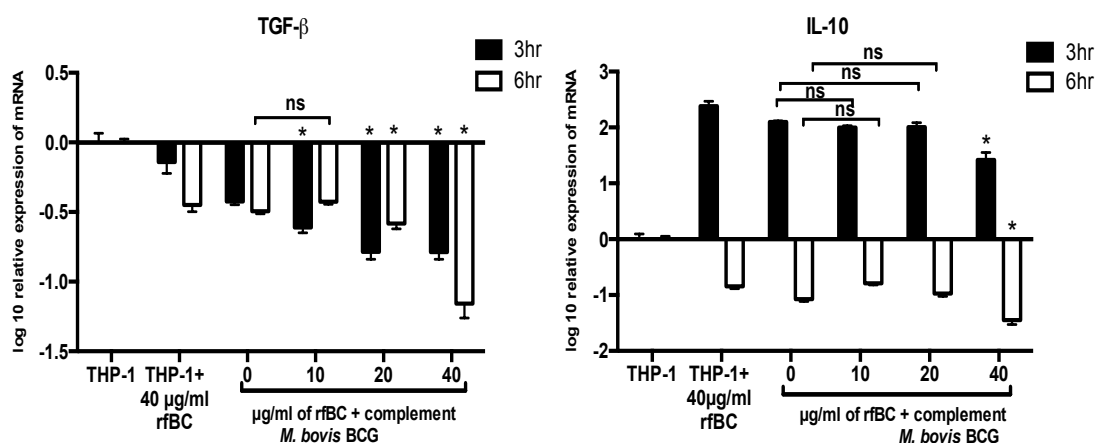
Quantitative real-time PCR (qPCR) was used to study the mRNA expression of pro-inflammatory (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12) cytokines (Figure 5.4). In this study we observed that rfBC-treated with complement deposited *M. bovis* BCG shows a down-regulation of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12, at both 3 and 6 hours of incubation with THP-1 macrophages.



**Figure 5.4: Expression of pro-inflammatory cytokines by THP-1 cells treated with complement deposited *M. bovis* BCG and different concentrations of rfBC.** qPCR analysis of the expression of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12) at 3 and 6 hours incubation of complement deposited *M. bovis* BCG and 10  $\mu$ g, 20  $\mu$ g and 40  $\mu$ g of rfBC. The calibrator sample used was THP-1 cells only. The data was normalized to 18S rRNA gene expression which was used as an endogenous control. The data are expressed as a mean of three independent experiments carried out in triplicates (SEM). The log RQ value were plotted to show the gene expression. There is a significance difference established between un-treated samples (THP-1 + complement deposited *M. bovis* BCG) and treated (THP1 + complement deposited *M. bovis* BCG + rfBC). The significance was revealed using the 2-way ANOVA test. All comparisons are statistically significant (p  $\leq$  0.05).

### 5.2.5 The anti-inflammatory response of THP-1 macrophages during phagocytosis of complement deposited *M. bovis* BCG treated with and without rfBC.

In this study we investigated the anti-inflammatory cytokine TGF- $\beta$  and IL10 gene expressions (Figure 5.5). We found TGF- $\beta$  showed an increase in expression in the presence of rfBC-treated *M. bovis* BCG. The graph shows that TGF- $\beta$  downregulated after 3 and 6 hours of the infection in the presence of rfBC as compared to untreated complement deposited *M. bovis* BCG. IL-10, was also down-regulated during these initial hours of interaction. THP-1 cells incubated only with rfBC did not show any remarkable changes in cytokine expression.

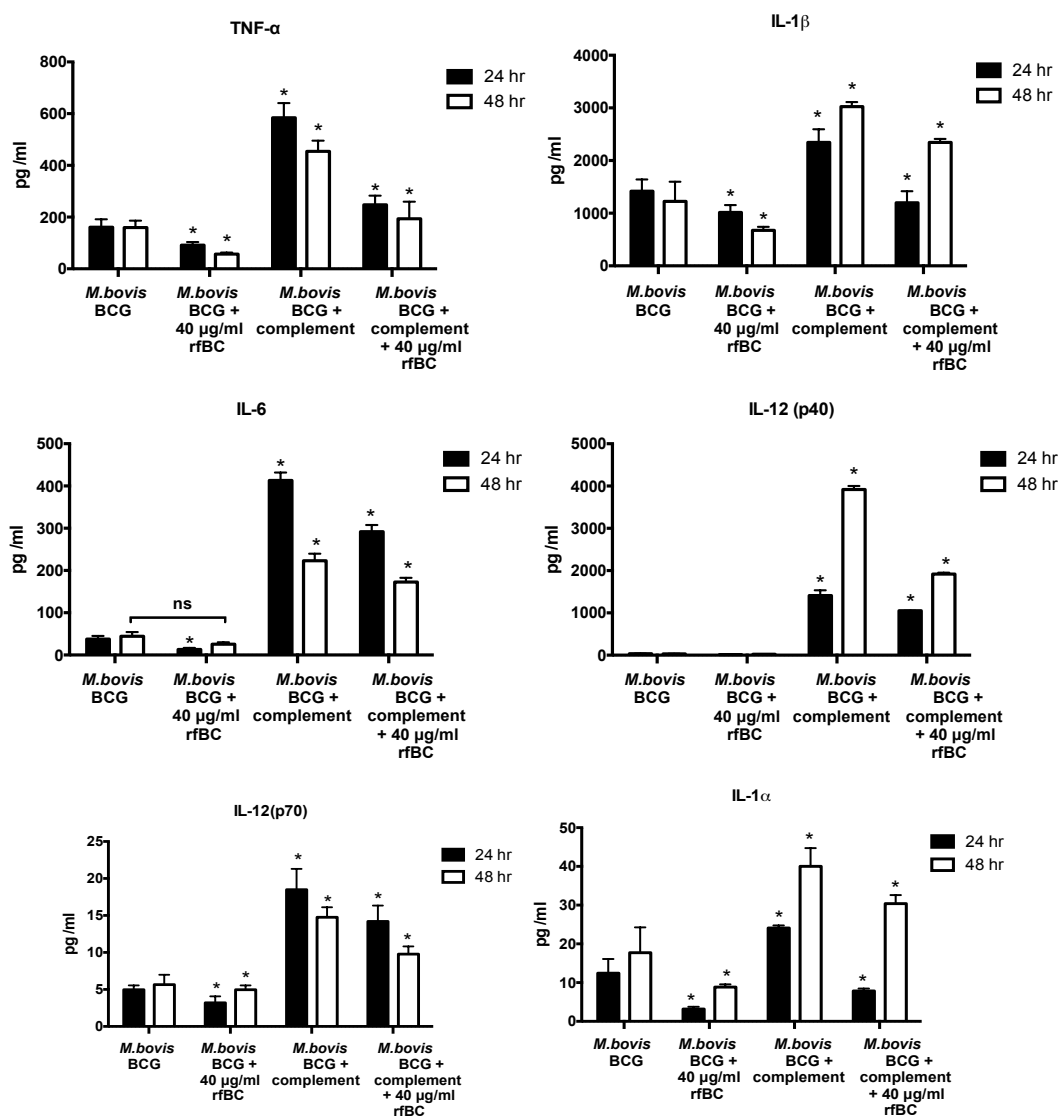


**Figure 5.5: Expression of anti-inflammatory cytokines by THP-1 cells treated with complement deposited *M. bovis* BCG and different concentrations of rfBC:** qPCR analysis of the expression of anti-inflammatory cytokines (TGF- $\beta$  and IL-10) at 3 and 6 hours incubation of *M. bovis* BCG and 10  $\mu$ g, 20  $\mu$ g and 40  $\mu$ g of rfBC. The calibrator sample used was THP-1 cells only. The data was normalized to 18S rRNA gene expression which was used as an endogenous control. The data are expressed as a mean of three independent experiments carried out in triplicates (SEM). The log RQ value were plotted to show the gene expression. There is a significance difference established between un-treated samples (THP-1 + complement deposited *M. bovis* BCG) and treated (THP1 + complement deposited *M. bovis* BCG + rfBC). The significance was revealed using the 1-way ANOVA test. All comparisons are statistically significant ( $p \leq 0.05$ ), except where shown (ns: not significant).

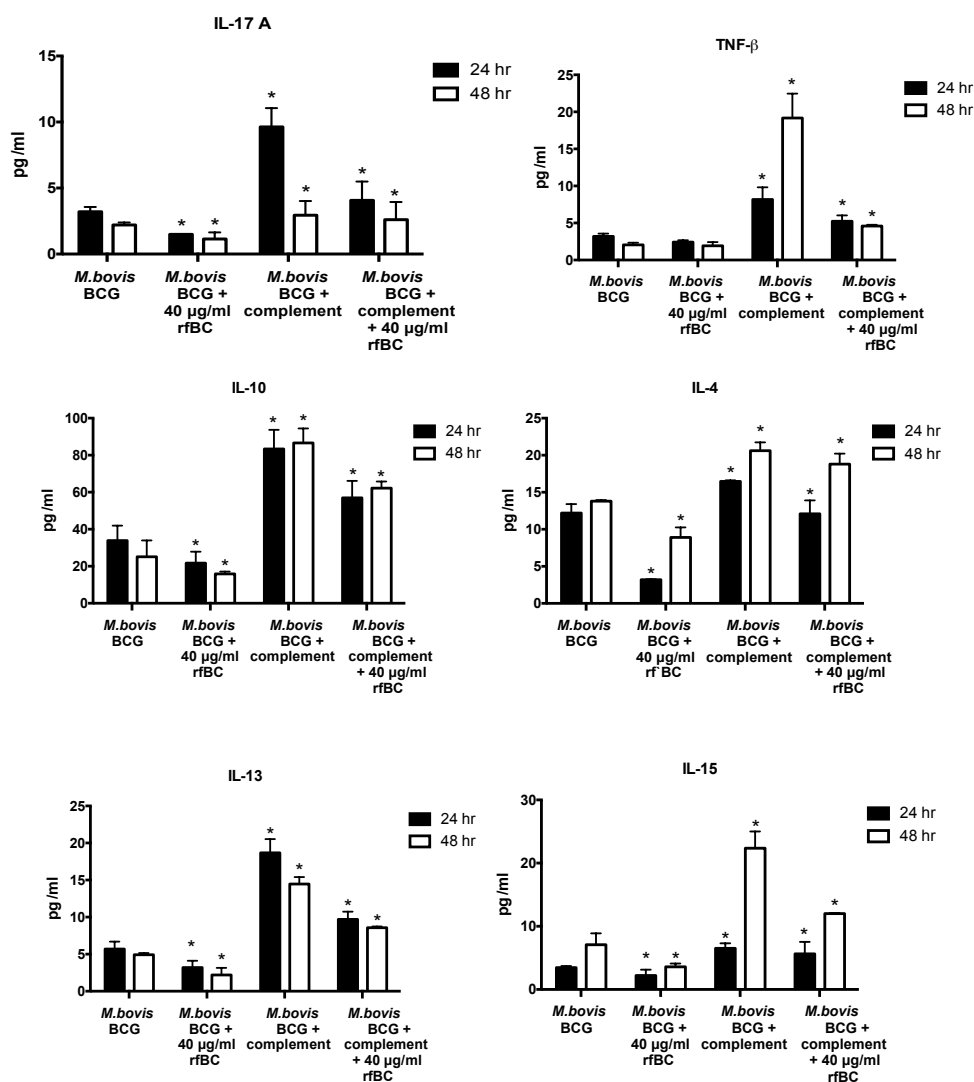
## 5.2.6 Multiplex array analysis to study cytokine, chemokine and growth factor production from THP-1 cells infected by *M. bovis* BCG treated with rfBC and complement and rfBC.

### 5.2.6.1 Production of pro-inflammatory and anti-inflammatory cytokines by THP-1 cells after 24 and 48 hours of the infection.

Multiplex analysis revealed that THP-1 cells infected with rfBC treated complement deposited *M. bovis* BCG down regulated the level of cytokines TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40 as compared to THP-1 cells infected with untreated complement deposited *M. bovis* BCG after 24 and 48 hours. Further suggesting a dampening of pro-inflammatory responses.



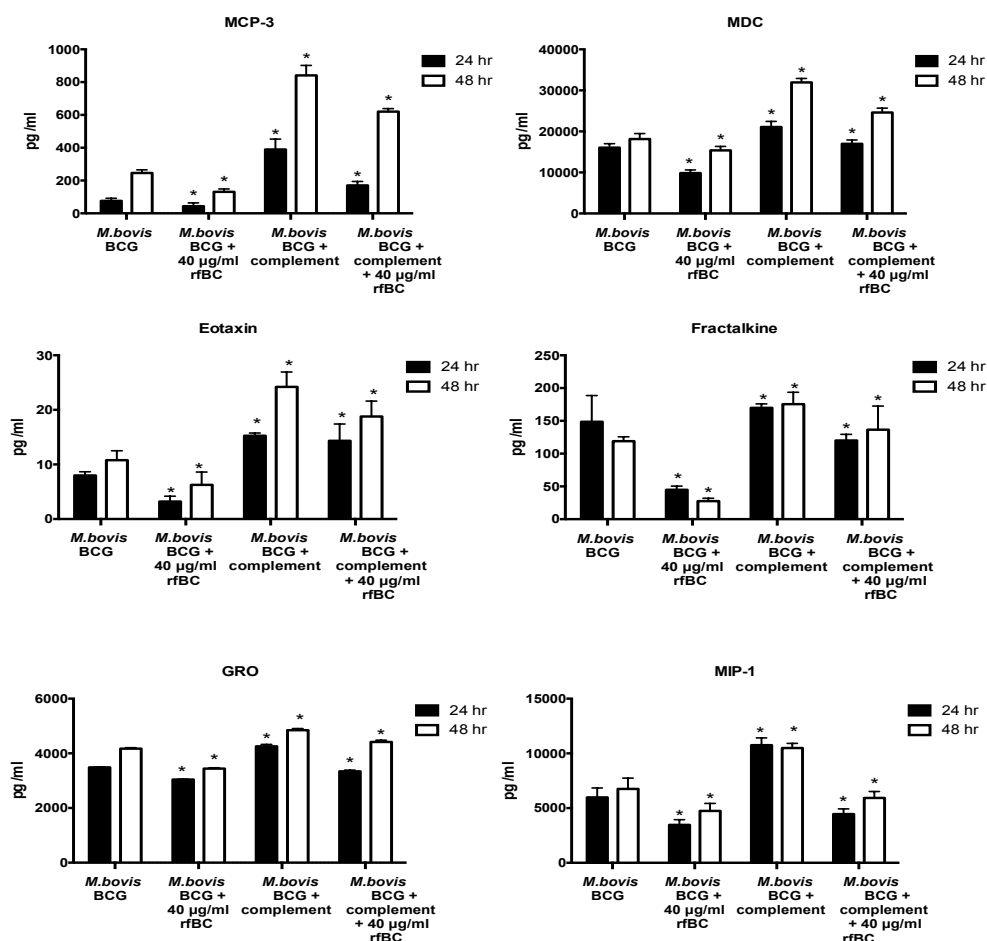




**Figure 5.6: Multiplex cytokine analysis of supernatants collected at 24 h after phagocytosis of complement deposited *M. bovis* BCG by THP-1 macrophages treated with/without rfBC.** *M. bovis* BCG deposited with complement coated with rfBC were incubated with THP-1 cells for 24 h and 48 h. Supernatant from late time points (24 and 48 h) (X-axis) were used for the measurement of the levels of cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12 (p40,p70), IL-1 $\alpha$ , IL-17 and TNF- $\beta$ , IL-10, IL-4, IL-13 and IL-15) inside the THP-1 by MagPix Milliplex kit (EMD Millipore). Assays were performed in duplicate. Bars represent  $\pm 2$  standard deviations. A one-way ANOVA was performed to determine significant differences in expression between *M. bovis* BCG (untreated) and *M. bovis* BCG coated with rfBC; *M. bovis* BCG (untreated) to *M. bovis* BCG + complement; *M. bovis* BCG + complement to *M. bovis* BCG complement coated with rfBC of 24 and 48 hours. All these comparisons were significant ( $P < 0.05$ ), except where shown [not significant (ns),  $P > 0.05$ ].

### 5.2.6.2 Production of chemokines by THP-1 cells after 24 and 48 hours of the infection.

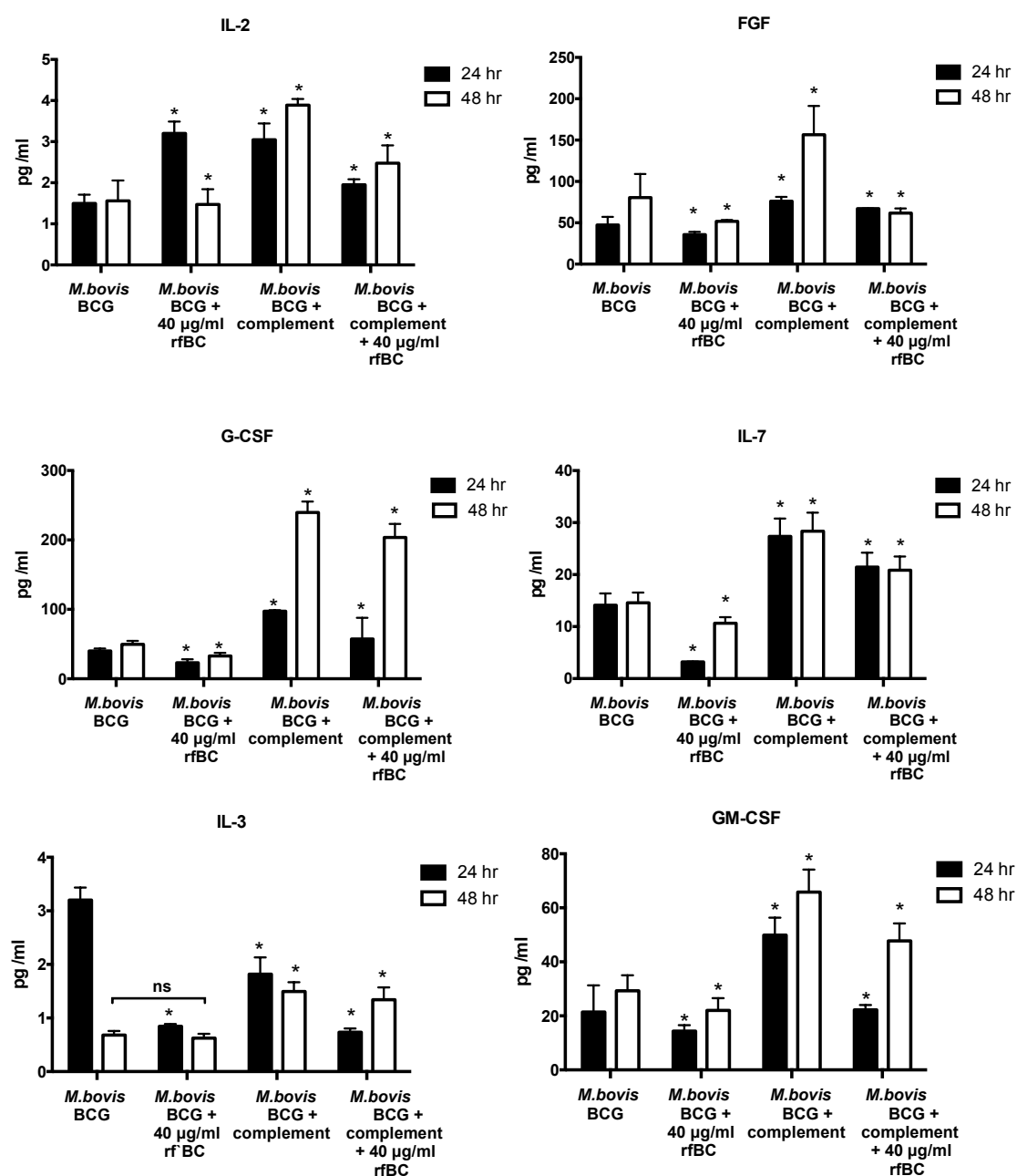
Multiplex analysis revealed that THP-1 cells infected with complement and rFBC treated *M. bovis* BCG down regulated the level of chemokines as compared to THP-1 cells infected with untreated complement coated *M. bovis* BCG after 24 and 48 hours.

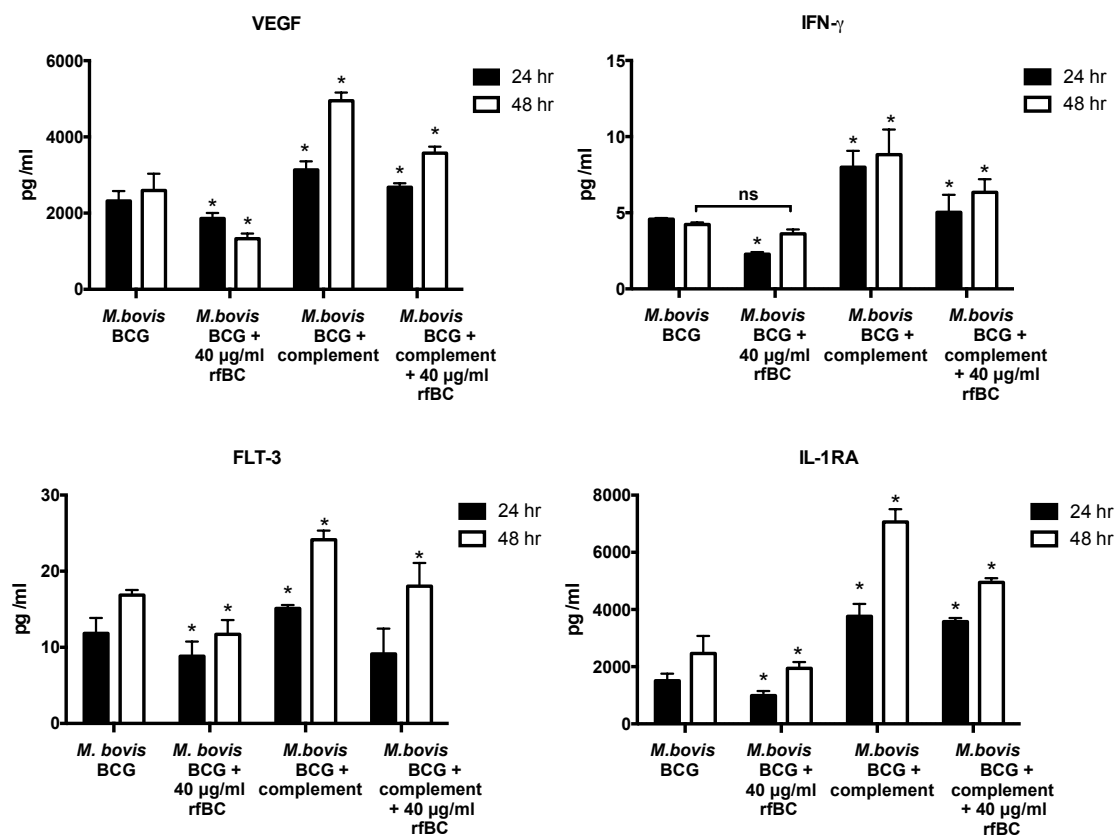


**Figure 5.7: Multiplex chemokines analysis of supernatants collected at 24 h after phagocytosis of complement deposited *M. bovis* BCG by THP-1 treated with rFBC.** Supernatant from late time points (24 and 48 h) (X-axis) were used for the measurement of the levels of chemokines (MCP-3, MDC, Eotaxin, Fractalkine, GRO and MCP-1) inside the THP-1 by MagPix Milliplex kit (EMD Millipore). Assays were performed in duplicate. Bars represents  $\pm 2$  standard deviations. A one-way ANOVA was performed to determine significance differences in expression between *M. bovis* BCG (untreated) and *M. bovis* BCG coated with rFBC; *M. bovis* BCG (untreated) to *M. bovis* BCG + complement; *M. bovis* BCG + complement to *M. bovis* BCG complement coated with rFBC of 24 and 48 hours. All these comparisons were significant ( $P < 0.05$ ).

### 5.2.6.3 Production of growth factors and related ligands and receptors production by THP-1 cells after 24 and 48 hours of the infection.

In this study multiplex analysis shown that THP-1 cells infected with rfBC and complement coated *M. bovis* BCG down regulated the level of growth factors and related ligands and receptors as compared to THP-1 cells infected with untreated *M. bovis* BCG after 24 and 48 hours.

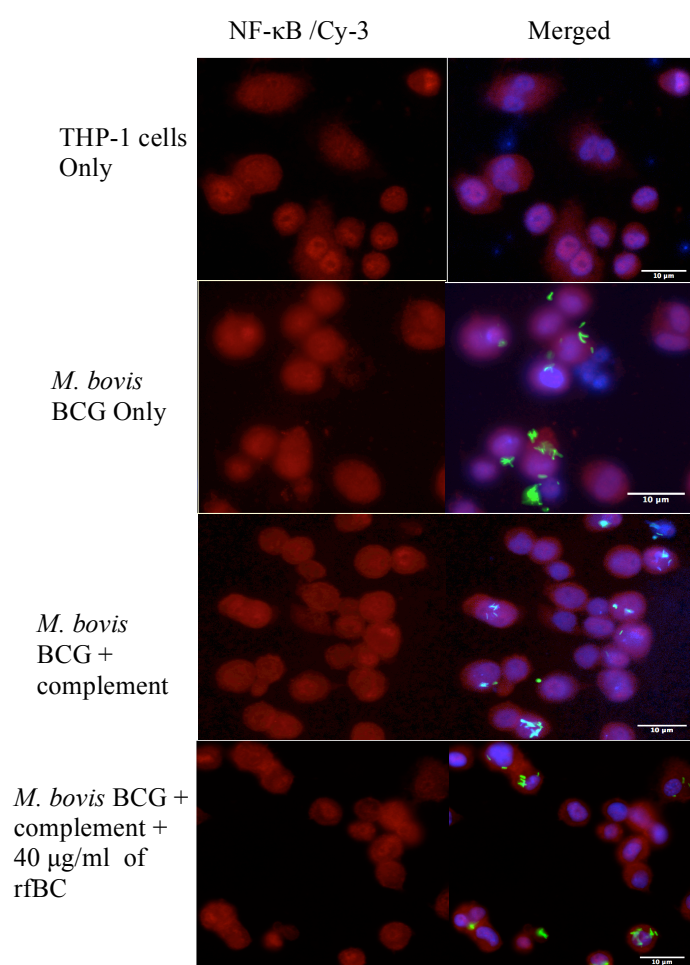




**Figure 5.8 : Multiplex analysis of growth factors and related ligands and receptors supernatants collected at 24 and 48h after phagocytosis of complement deposited *M. bovis* BCG by THP-1 macrophages treated with rfBC.** Supernatant from time points (24 and 48 h) (*X*-axis) were used for the measurement of the levels of growth factors (IL-2, FGF-2, G-CSF, IL-7, IL-3, GM-CSF and VEGF) and related ligands and receptors (IFN- $\gamma$ , FLT-3 and IL-1RA) inside the THP-1 by MagPix Milliplex kit (EMD Millipore). Assays were performed in duplicate. Bars represents  $\pm 2$  standard deviations. A one-way ANOVA was performed to determine significance differences in expression between *M. bovis* BCG (untreated) and *M. bovis* BCG coated with rfBC; *M. bovis* BCG (untreated) to *M. bovis* BCG + complement; *M. bovis* BCG + complement to *M. bovis* BCG complement coated with rfBC of 24 and 48 hours. All these comparisons were significant ( $P < 0.05$ ).

### 5.2.7 Production of rfBC treated complement deposited *M. bovis* BCG on NF- $\kappa$ B cytoplasm to nucleus translocation.

In this study we investigated the effect of rfBC on the translocation of NF- $\kappa$ B in THP-1 cells infected with complement coated *M. bovis* BCG. We performed this analysis by using fluorescence microscopy. We observed that NF- $\kappa$ B translocation was more prominent in complement-deposited *M. bovis* BCG and untreated *M. bovis* BCG. However, there was no significant translocation observed in rfBC-bound complement deposited *M. bovis* BCG (Figure 5.13).



**Figure 5.9: NF- $\kappa$ B translocation in THP-1 cells after uptake of *M. bovis* BCG treated with rfBC and complement plus rfBC.** Fluorescence microscopy was performed to investigate the effect of rfBC the translocation of Nf- $\kappa$ B to nucleus in THP-1 cells after the phagocytosis of complement deposited GFP *M. bovis* BCG. Complement deposited GFP *M. bovis* BCG used as a control. Cells were treated with rabbit anti Nf- $\kappa$ B p65 (1:200) primary antibodies followed by Cy3-conjugated goat anti-rabbit antibody (red). THP-1 cell nucleus is stained with Hoechst (blue) (1:10000)

### 5.3 Discussion

Outer membrane of mycobacteria is composed of peptidoglycan layer that is covalently attached to the arabinogalactans, arabinomannans, glycolipids and mycolic acids (Daffe and Draper, 1997). In mycobacteria extra capsule layer is present on the top of outer membrane, this capsule contains polysaccharides, proteins and lipids. These lipids comprised of mostly phospholipids and glycolipids (Daffe and Etienne, 1999). Cord factor or trehalose dimycolate is a glycolipid present in the cell wall of mycobacteria and is responsible to activate the complement (Ramanathan *et al.*, 1980). Complement system plays an important role into the innate immunity by activating three different pathways. Mycobacterium is intracellular pathogen that entered and multiplies in to the host cells. In one of the study it has been shown that *M. bovis* BCG have ability to activate classical and alternative pathways. In the same study it has been demonstrated that MBL and L-ficolin binds to the mycobacterial surface result in to the activation of lectin pathway (Carrol *et al.*, 2009). Complement activation by any pathways resulted in to the C3b deposition on the mycobacterial surface (Carrol *et al.*, 2009).

By reproducing the complement assay done by Carrol *et al.* (2009), this study confirmed that *M. bovis* BCG is capable of activating complement through alternative pathway (Carrol *et al.*, 2009) Based on what is observed in this study, with the consumption of different bacterial concentrations. The assay was optimised and for the data presented in this study, for which serum was kept on ice while the samples were incubated for complement consumption. Therefore, the integrity of the serum was preserved and assessed after the haemolytic assay performance.

In this study when mycobacteria are coated with complement, the uptake of *M. bovis* BCG increased by THP-1 cells, but after incubation of complement coated *M. bovis* BCG with rfBC, rfBC dose dependently inhibited *M. bovis* BCG uptake by THP-1 cells. It is known that C3b is an opsonin, enhancing uptake of *M. tuberculosis* by human alveolar and monocyte-derived macrophages (Schlesinger et al 1990). Due to genomic similarities of *M. bovis* BCG to *M. tuberculosis*, it would be likely that C3 proteins enhanced *M. bovis* BCG uptake by THP-1 cells. It has been shown that mycobacteria could utilise complement opsonisation to be phagocytised by macrophages to escape from immune system and remained in the phagosome of

macrophages. It has been shown that the deposition of C3b on pathogen surface is the end result of complement activation by any pathway. Factor H is known as a main regulator of complement activation, which act as a cofactor for factor I to cleaves the  $\alpha$ -chains of C3b to generate in active form known as iC3b (Sim *et al.*, 1993). It has been known that conglutinin has the ability to agglutinate the erythrocytes those had been reacted with antibodies and complement in the presence of factor I (Lachmann and Muller, 1968). In the previous studies it has been shown that conglutinin binds with C3 fragment (iC3b) in the presence of calcium ions (Leon, 1957; Ross, 1986).

It has been shown that C3b deposition on the outer surface of mycobacteria helps in the phagocytosis by complement receptors (CR1, CR3 or CR4) present on the macrophages (Ferguson *et al.*, 2004; Hetland and Wiker, 1994; 2001; Schlesinger and Horwitz, 1994). C3b binding to *M. tuberculosis* and *M. bovis* BCG has been shown to occur via classical and alternative complement pathways and is present in the form of C3b and iC3b (Carroll *et al.*, 2009, Ferguson *et al.*, 2004). C3b is important component taking part in the complement pathways to develop towards the membrane attack complex (MAC), but iC3b is an inactivate component of C3 does not take part in this reaction. C3b is known as a ligand for complement receptor 1 (CR1) but iC3b is ligand for complement receptor 3 and 4 (CR3 and CR4) (Ross, 1986). However, it has not been demonstrated how these complement pathways are initiated by mycobacteria. In this study, we found that complement deposition on *M. bovis* BCG enhanced the uptake of bacterium by THP-1 cells. Although complement-deposited *M. bovis* BCG resulted in enhanced uptake by 22% as compared to untreated *M. bovis* BCG but rfBC significantly inhibits phagocytosis of mycobacterium by THP-1 cells. This inhibition in phagocytosis by rfBC to be dose-dependent, there is 44% reduction in uptake observed for 10  $\mu\text{g/ml}$ , 55% for 20  $\mu\text{g/ml}$  and 83% for 40  $\mu\text{g/ml}$  of rfBC as compared to untreated complement coated *M. bovis* BCG. The reason for less uptake of complement coated mycobacteria by THP-1 cells in presence of rfBC could be as follows: Since rfBC is a known binder of iC3b (Ross, 1986), it may therefore bind to iC3b deposited on the *M. bovis* BCG surface, and inhibited the uptake of mycobacteria by complement receptors CR3 and CR4 present on the macrophages.

In this study we observed the pro and anti-inflammatory cytokines role of rfBC and complement in shaping the adaptive immune response during host-pathogen

interaction. In our findings, rfBC and complement do seem to modulate cytokine responses during uptake of *M. bovis* BCG by THP-1 cells.

TNF- $\alpha$  expression was down regulated by rfBC in the early stages of phagocytosis of complement coated *M. bovis* BCG as compared to untreated complement coated *M. bovis* BCG. The level of mRNA expression of TNF- $\alpha$  was down regulated after three hours and six hours of the infection. Multiplex analysis of 24 and 48 hours revealed the down regulation of TNF- $\alpha$  response was consistent with early hours response. It has been studied that TNF- $\alpha$  stimulate the formation of granuloma (Flesch and Kaufmann, 1990). TNF- $\alpha$  is an important cytokine produced in the early stages of infection; play an important role in the macrophages activation and granuloma formation (Flesch and Kaufmann, 1990). It has been shown that TNF- $\alpha$  helps in the maintaining of latent tuberculosis infection in the mice model by granuloma formation (Flynn *et al.*, 1995).

IL-1 $\beta$  is another important cytokine play a role against human tuberculosis infection in the host (Mayer-Barber *et al.*, 2010). It is produced by immune cells, such as monocytes, macrophages and dendritic cells in the response to *M. tuberculosis* (Giacomini *et al.*, 2001). We examined the down regulation of IL-1 $\beta$  in the presence of rfBC complement deposited *M. bovis* BCG in 3 and 6 hours of the infection as compared to untreated complement coated *M. bovis* BCG. Multiplex analysis of 24 and 48 hours revealed the same result of down regulation of IL-1 $\beta$  response in the rfBC treated complement deposited *M. bovis* BCG as compared to untreated, which was consistent with early responses.

NF- $\kappa$ B is an important transcription factor that regulates the synthesis of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ . We studied the NF- $\kappa$ B translocation in THP-1 cells stained with an antibody against the NF- $\kappa$ B and analysed by fluorescence microscope. We observed no nuclear translocation of NF- $\kappa$ B in response to rfBC bound complement coated *M. bovis* BCG as compared to untreated complement coated *M. bovis* BCG. This result is further evidence of a dampening of the inflammatory response in the presence of rfBC and also suggested the down regulation of pro-inflammatory cytokines and chemokines.

It has been shown that, IL-6 stimulates the T cell response against mycobacteria (Leal *et al.*, 1999). IL-6 produced by macrophages at the site of infection during initial stages



of the tuberculosis infection (Hoheisel *et al.*, 1998). In this study we observed the down regulation of IL-6 in the early stages after 3 and 6 hours of infection in the presence of rfBC. Multiplex analysis of 24 and 48 hours revealed the down regulation of IL-6 response was consistent with early 3 hours response.

IL-12 expression was down regulated in the presence of rfBC in early response of complement deposited *M. bovis* BCG as compared to untreated (*M. bovis* BCG + complement), and multiplex analysis of 24 and 48 hours revealed the same response, which may inhibit the Th1 response and produce INF- $\gamma$  reducing inflammation. The down regulation of IL-12 by rfBC may be directly lead to reduce the uptake of *M. bovis* BCG by macrophages. IL-12 suppression may result in to the down regulation of Th1 response and activation of the CD4<sup>+</sup> T cells, inducing INF- $\gamma$  production. It has been shown that T-cells activation can stimulate the granuloma necrosis (Ehlers *et al.*, 2001) and IL-12 down regulation could be important factor to disturb the balance of Th1/Th2 with in the granuloma. The down regulation of the pro-inflammatory cytokines response by rfBC may suppress the production of important cytokines and chemoattractants (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, IFN- $\gamma$ ) those are helpful in recruiting the immune inflammatory cell and also discourage inflammation and granuloma formation (Feng *et al.*, 2006). In vivo, macrophages phagocytose mycobacteria after entry in to the host but ultimately fail to destroy them and provide hostile environment for multiplication. rfBC inhibition of phagocytosis of these bacteria in to macrophages may be protective by keeping them extracellular where these can be easily cleared by the host immune response.

It has been studied that macrophages produced IL-10 in the early stages of tuberculosis infection, following the uptake of *M. tb* (Shaw *et al.*, 2000). It has also been reported that IL-10 prevents maturation of phagosome, this helps in the *Mycobacterium* survival in the macrophages (O'Leary *et al.*, 2011). It has been observed that IL-10 also inhibits dendritic cell activation (Demangel *et al.*, 2002) result in to the inhibition of Th1 response (Tian *et al.*, 2005). It has been seen that IL-10 and TGF- $\beta$  level was high in the lungs of active tuberculosis patient, which indicates the importance of these two cytokines in the pathogenesis of tuberculosis infection (Gong *et al.*, 1996). TGF- $\beta$  helps in the survival of mycobacteria by down regulating the immune cells. TGF- $\beta$  prevents T-cells maturation and also down regulating the important cytokines (IL-1,

IL-6, TNF- $\alpha$ ) those can recruit inflammatory cells for granuloma formation (Toossi and Ellner, 1998). We observed in this study, IL-10 and TGF- $\beta$  were down regulated at early stages of phagocytosis in the presence of rfBC, suggesting the inhibition of anti-inflammatory cytokines response. The down regulation of these two important cytokines may help in the clearance of mycobacteria in the early stages of infection and protect the host from further progression of infection. Multiplex analysis of 24 and 48 hours revealed the down regulation of IL-10 response, which is consistent with early response. In this study the cytokines findings may reflect the initial inflammatory response in vivo, which lead to granuloma formation to prevent the further growth of mycobacteria and also gives a cavity to mycobacteria to stay as a latent infection. It has been shown that balance of inflammation is required to maintain the granuloma. The balance of inflammation is achieved by maintaining the balance in pro-inflammatory (IFN- $\gamma$ , TNF- $\alpha$ ) compared to anti-inflammatory (IL-10, IL4, TGF- $\beta$ ) cytokines within granuloma (Ehlers and Schaible, 2013).

We observed the down regulation of IL-4 when THP-1 cells treated with complement deposited *M. bovis* BCG bound with rfBC as compared to untreated complement deposited *M. bovis* BCG. IL-4 is an anti-inflammatory cytokine, which down regulates the production of IFN- $\gamma$  and has a negative impact on the activation of macrophages (Lucey *et al.*, 1996). It has been shown that mice infected with tuberculosis showed increased production of IL-4, which can help in the disease development and reactivation of latent infection (Hernandez-Pando *et al.*, 1996). In another study, it has been shown that after treatment of IL-4 by anti-IL-4 antibody reduced the *M. tb* pathogens in the lungs of the mice during the initial stages of infection as compared to control. In the same study IL-4 gene knock out mice showed the less number of *M. tb* in the lungs and spleen tissues (Buccheri *et al.*, 2007). By giving exogenous IL-4 doses to these mice resulted in the higher number of *M. tb* in the host, suggested that IL-4 helps in the survival of mycobacteria in the host (Buccheri *et al.*, 2007).

We also examined the chemokines response from THP-1 cell lines treated with complement deposited *M. bovis* BCG with and without rfBC coating. In the previous chapter we observed the chemokines response from THP-1 cell lines without complement deposited *M. bovis* BCG bound with rfBC and *M. bovis* BCG only. We observed that recombinant rfBC treated complement deposited *M. bovis* BCG down

regulated the level of chemokines (MCP-3, MDC, Eotaxin, Fractalkine, GRO and MCP-1) by infecting THP-1 cells after 24 and 48 hours as compared to untreated complement deposited *M. bovis* BCG. Monocyte chemo-attractant protein-1 (MCP-1) increased Th2 polarization and attracts immune cells to the site of infection (Rose *et al.*, 2003). Monocyte chemo-attractant protein-3 (MCP-3) is secreted by macrophages and reported higher level in bronchoalveolar fluid of tuberculosis patient (Ruhwald *et al.*, 2009). Growth-related oncogene-1 (GRO1) also produced by macrophages, neutrophils and epithelial cells. GRO assist in the recruitment of neutrophils and monocytes to the infectious site (Nakagawa *et al.*, 1994). Macrophage derived chemokine (MDC) is also helping in the recruitment of immune cells such as monocytes, dendritic cells and natural killer cells to the site of infection (Godiska *et al.*, 1997). The down regulation of chemo attractants response by rfBC discourage the inflammation and granuloma formation process and also prevent them to be phagocytosed by macrophages where they can multiply and survive.

In this study we studied the level of growth factors and related ligands and receptors by THP-1 cells treated with complement deposited *M. bovis* BCG bound with rfBC as compared to complement deposited *M. bovis* BCG after 24 and 48 hours. We observed the down regulation of growth factors (IL-2, FGF-2, G-CSF, GM-CSF and VEGF) and related ligands and receptors (IFN- $\gamma$ , FLT-3 and IL-1RA) by THP-1 cells treated with complement deposited *M. bovis* BCG bound with rfBC as compared to complement deposited *M. bovis* BCG after 24 and 48 hours. Granulocytes-colony stimulating factor (G-CSF) is one of the important growth factor produced by monocytes, fibroblasts and endothelial cells (Schneider *et al.*, 2005). It encourages the immunological functions of neutrophil by differentiating into mature neutrophil from the precursors (Hu and Yasui, 1997).

In this study we studied the IFN- $\gamma$  response from THP-1 cells lines treated with complement deposited *M. bovis* BCG with and without rfBC coating. We observed that rfBC treated complement deposited *M. bovis* BCG down regulated the level of IFN- $\gamma$ . It has been shown that IFN- $\gamma$  mainly produced by Natural killer (NK) cells, CD4<sup>+</sup>, CD8<sup>+</sup>, macrophages and mucosal epithelial cells (Munder *et al.*, 2001). It has also been observed that mice with IFN- $\gamma$  deficient genes were able to form granuloma (Flynn *et al.*, 1993).

The pro-inflammatory response was raised significantly in complement-deposited *M. bovis* BCG and complement-deposited *M. bovis* BCG treated with rfBC and were at similar levels to *M. bovis* BCG only. However, for complement-deposited *M. bovis* BCG treated with rfBC, there was significant inhibition of phagocytosis, whilst for complement-deposited *M. bovis* BCG there was an enhancement of uptake.

The dampening of the pro-inflammatory response by conglutinin may also suppress the production of important cytokines and chemoattractants (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40) that recruit inflammatory cells and discourage inflammation and tissue remodeling necessary for granuloma formation (Feng *et al.*, 2006; Mayer-Barber *et al.*, 2010). In the absence of complement in the lungs, this may be a preferred strategy by the host to protect against mycobacterial infection. However, there is evidence of complement in the lungs that could support C3 deposition on mycobacteria (Ferguson *et al.*, 2004). In this scenario, conglutinin could be acting once again to maintain the pathogen in the extracellular milieu, while also simultaneously tailoring the enhanced pro-inflammatory response to the extracellular environment for clearance of the pathogen.

## **Chapter 6: Concluding Discussion and Future perspectives**

## 6.1 Conclusions and future perspectives

In this study, bovine conglutinin was investigated for its host-pathogen interactions with mycobacteria *in vitro*. A recombinant fragment of conglutinin (rfBC) was expressed and purified containing the carbohydrate recognition domain (CRD) and neck region. The binding and direct effect of rfBC on the growth of mycobacteria was investigated, together with the effect of rfBC on phagocytosis of mycobacteria by macrophages. The effect of rfBC on the phagocytosis of complement deposited *M. bovis* BCG was also investigated. In the studies conducted, *M. bovis* BCG and *M. smegmatis* (non-pathogenic mycobacterial species) were used as model organisms of *M. bovis* and *M. tuberculosis*, respectively, to demonstrate that rfBC was able to bind to mycobacteria and play an important role on their uptake by macrophages. These studies show that rfBC interactions with mycobacteria and macrophages can modulate the subsequent inflammatory response, possibly influencing the outcome of adaptive immunity. In this chapter, the possible implications from our results will be discussed and with respect to the understanding of rfBC role in the bovine tuberculosis and how it may rfBC could be used as a possible treatment, prophylactic or biomarker against bovine and possibly human tuberculosis.

Conglutinin is an important member of collectin family, which is synthesized in the liver and is present in the serum and can be produced locally by neutrophil, dendritic and macrophage cells (Dec *et al.*, 2012). Previously conglutinin has been shown to possess growth inhibition activity against Gram-negative bacteria and also has antiviral effects against influenza A virus and human immunodeficiency virus (HIV) (Laursen *et al.*, 1994). Bovine tuberculosis remains an important agricultural economic problem in the United Kingdom and worldwide, it is not only effecting cattle but also found in domestic and wild animals including humans (Abernethy *et al.*, 2013). Conglutinin is found in the cattles, which makes an important collectin to be studied in the bovine tuberculosis (*M. bovis* infection). However, its role in bovine tuberculosis has never been studied before. The primary organ targeted by *M. bovis* is the host lung and the bacterial infection is accompanied by a localized inflammation, resulting in the migration of different immune cells and leakage of serum proteins on the site of infection. Some of the first host molecules to be involved are collectins, which are soluble innate immune molecules that play an important role in targeting

microbes by binding to oligosaccharides and lipid structures on their surface and can stimulate aggregation, complement activation, opsonisation, effect on phagocytosis and inhibit growth (Hartshorn *et al.*, 1993; Reading *et al.*, 1997; Wang *et al.*, 1995).

It is hypothesized that during inflammation conglutinin leaks from the blood vessels at the site of infection due to changes in vascular permeability and is likely to come in direct contact with *M. bovis* and immune cells and therefore may modulate the outcome of the infection. It is also possible that alveolar macrophages may also produce conglutinin locally. It has been studied that conglutinin binds with zymosan and complement fragment iC3b in the presence of calcium ions and also shows antimicrobial activity *in vitro* (Dec and Wernicki 2006).

I observed the rfBC binds to *M. bovis* BCG and *M. smegmatis* and this binding is dose-dependent, reaching saturation of binding at 20µg/ml. This binding of rfBC with *M. bovis* BCG and *M. smegmatis* was greater in the presence of Ca<sup>2+</sup> as compared to EDTA. This is the first observation of conglutinin binding to a Gram-positive bacterium (*M. bovis* BCG and *M. smegmatis*). We observed less binding between rfBC with *M. smegmatis* as compared to *M. bovis* BCG. As rfBC is a bovine protein and *M. bovis* is a causative agent of bovine TB, so there should be some relevance in the binding. Surfactant protein D (SP-D) has also been shown to bind to the mannose caps of *M. tuberculosis* lipoarabinomannan (LAM) and other carbohydrates present on the surface of *M. tuberculosis* (Ferguson, 2002), as SP-D is mostly structurally similar to conglutinin (Hansen and Holmskov, 2002) and *M. bovis* BCG has similar bacterial surface as *M. tuberculosis* including LAM (Jankute *et al.*, 2014; Jungblut *et al.*, 1999), so most likely the ligand for rfBC interaction with *M. bovis* BCG is LAM.

We also observed that rfBC directly inhibits the growth of *M. bovis* BCG and *M. smegmatis* *in vitro* and we found this inhibition was dose-dependent. We also observed that rfBC inhibition is bacteriostatic in nature rather than bactericidal as our experiment shown that there was no cell lysis observed in rfBC treated *M. bovis* BCG. This inhibition could be through intracellular signaling in the mycobacteria to affect the important genes responsible for cell metabolism and bacterial survival. To the best of our knowledge the direct bacteriostatic inhibition role of collectin (conglutinin) on Gram-positive bacterium (mycobacteria) has not been studied before. In the previous studies it has been shown that SP-A and SP-D inhibited the growth of Gram-negative

bacteria by increasing the cell permeability but this role was also not bactericidal (Hogenkamp *et al.*, 2007; Wu *et al.*, 2003). In the previous studies, it has been shown that rfBC played an important role in the survival of mice infected with pathogen strains of *Salmonella typhimurium*, *Pasteurella septica*, *Klebsiella pneumoniae*, *Listeria monocytogenes* and *Streptococcus pyogenes* (Ingram 1959). It has been reported that rfBC helps in the prolonged survival of mice infected with *S. typhimurium* (Friis-Christiansen 1990).

In this study, we found that rfBC inhibits the phagocytosis and growth of mycobacteria (*M. bovis* BCG and *M. smegmatis*) inside the THP-1 cells in a dose dependent manner. Our data showed less uptakes of *M. smegmatis* by THP-1 cells as compared to the phagocytosis of *M. bovis* BCG. From previous findings we observed less bindings of *M. smegmatis* with rfBC as compared to *M. bovis* BCG binds to rfBC. This could be a reason of less uptake of *M. smegmatis* by THP-1 cells in the presence of rfBC. The inhibitory effect of rfBC was further confirmed by fluorescence microscopy. This reduction in uptake of mycobacteria (*M. bovis* BCG and *M. smegmatis*) in the presence of rfBC may stimulate intracellular signaling in the mycobacteria to affect the important genes function those are responsible for cell metabolism and bacterial survival. Other reason of the less uptake of mycobacteria by the THP-1 cells could be, rfBC bound with mycobacterial surface antigens, in the result less binding of mycobacteria to the mannose receptors on macrophages. The reason of reduced uptake could be rfBC bound to the bacterial cell wall component LAM, which are ligands for mannose receptors (Ferguson *et al.*, 2002). Further studies are required to confirm this highly likely possibility by performing conglutinin lectin-blotting to identify the mycobacterial ligand for conglutinin. This study can be performed by separating the cell wall component with the help of SDS-PAGE and transferred to the nitrocellulose membrane. This membrane will be incubated with conglutinin and further treated with the specific antibody of LAM to determine the conglutinin-LAM interaction. It has been reported that SP-D is highly similar to the bovine conglutinin and strongly binds to LAM of virulent *M. tuberculosis*, strains (Ferguson *et al.*, 2002). SP-D bound to *M. tuberculosis* has already been shown to be inhibiting binding of mycobacteria to human macrophages (Ferguson *et al.*, 2002).



The effect of complement and how conglutinin may impact the *M. bovis* BCG interaction with macrophages (THP-1) was also investigated. It has been shown that C3b deposition on the surface of mycobacteria enhances uptake of the bacterium by complement receptors (CR1, CR3 or CR4) present on the macrophages (Cywes *et al.*, 1996; Ferguson *et al.*, 2004; Hetland and Wiker, 1994; 2001; Schlesinger and Horwitz, 1994). Studies have shown C3 activation and deposition of C3 fragments onto *M. tuberculosis* and *M. leprae* cell surfaces via both the classical and alternative pathways (Ferguson *et al.*, 2004; Ramanathan *et al.*, 1980; Schlesinger and Horwitz, 1994). In another study, C3 binding to *M. tuberculosis* and *M. bovis* BCG has been shown to occur via classical and alternative complement pathways and is present in the form of C3b and iC3b (Carroll *et al.*, 2009, Ferguson *et al.*, 2004). C3b is important component taking part in the complement pathways to develop towards the membrane attack complex (MAC), but iC3b is an inactive component of C3 does not take part in this reaction. C3b is known as a ligand for complement receptor 1 (CR1) but iC3b is ligand for complement receptor 3 and 4 (CR3 and CR4) (Ross, 1986). In this study, we observed less uptake of complement deposited *M. bovis* BCG by THP-1 cells when treated with rfBC. The reasons for less uptake of complement coated *M. bovis* BCG by THP-1 cells in presence of rfBC could be; as conglutinin is a known unique binder of iC3b (Ross, 1986) and therefore conglutinin binds with iC3b deposited on the *M. bovis* BCG surface, and inhibits the uptake of mycobacteria through complement receptors CR3 and CR4 present on the macrophages. In this study we observed that there was increase in uptake of complement deposited *M. bovis* BCG by THP-1 cells as compared to *M. bovis* BCG only, this increase in phagocytosis likely to be enhanced uptake by complement receptors such as CR1, CR3 and CR4.

Overall, the summary of our findings from this thesis, show that conglutinin (rfBC) significantly inhibits phagocytosis of *M. bovis* BCG by macrophages (THP-1) in a complement dependent and complement independent manner by probably blocking the host-bacterial interaction to complement and mannose receptors. Further experiments need to be carried out to confirm the interaction of conglutinin with the mannose and complement receptors.

Furthermore, the role of conglutinin in shaping the macrophage pro- and anti-inflammatory response during mycobacterial infection was also investigated. The data show that conglutinin and complement do seem to modulate cytokine responses during phagocytosis of *M. bovis* BCG by THP-1 cells. We observed that conglutinin bound to *M. bovis* BCG cause the down regulation of the pro-inflammatory response after the initial 3 and 6 hours of phagocytosis as well as later hours 24 and 48 of the phagocytosis as compared to *M. bovis* BCG only. In this study we observed the up-regulation of pro-inflammatory cytokines in the THP-1 cells infected with complement deposited *M. bovis* BCG and complement deposited *M. bovis* BCG treated with rfBC led down regulation of cytokines in the THP-1 cells. In vivo, macrophages phagocytose mycobacteria but ultimately fail to destroy them and provide hostile environment for multiplication. rfBC inhibition of phagocytosis of these bacteria into macrophages may be protective against infection by keeping them extracellular where these can be easily cleared by the host immune response (Feng *et al.*, 2006). The dampening of the pro-inflammatory response by conglutinin may also suppress the production of important cytokines and chemoattractants (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40) that recruit inflammatory cells and discourage inflammation and tissue remodeling necessary for granuloma formation. In the absence of complement in the lungs, this may be a preferred strategy by the host to protect against mycobacterial infection. However, there is evidence of complement in the lungs that could support C3 deposition on mycobacteria. In this scenario, conglutinin could be acting once again to maintain the pathogen in the extracellular milieu, while also simultaneously tailoring the enhanced pro-inflammatory response to the extracellular environment for clearance of the pathogen.

Conglutinin is secreted by liver and found in the serum of the bovine host. The mean serum concentration of the conglutinin is 12  $\mu\text{g/ml}$  in the serum (Holmskov *et al.*, 1998), which is approximately similar to the concentration of rfBC used in this study. Low levels of serum concentration of conglutinin have been reported in cattle suffering from respiratory infection (Holmskov *et al.*, 1998). There are very few cases reported of mycobacterium presence in the cattle plasma (Lepper *et al.*, 1977; Srivastava *et al.*, 2008), this is due to either underreporting of these cases, due to culling, or perhaps cattle are better able to cope with disseminated infections (Swift *et al.*, 2016).

There is no evidence of conglutinin being produced locally in the bovine lungs. One previous study showed its presence in the lung macrophages, but it is not clear either this conglutinin that this conglutinin travelled from other part of the body or if it is secreted in the lung macrophages (Holmskov *et al.*, 1992). Further studies are required to find out whether conglutinin is present in the lungs. If the presence of the conglutinin is confirmed in the lungs, then it could play a significant role in the early stages of tuberculosis infection. In the mycobacterial infection, factors enhance the phagocytosis of bacterium in to the macrophages are likely to be hazardous to the host and can help in the pathogenesis of disease. In this study we found that rfBC inhibit the phagocytosis of *M. bovis* BCG and *M. smegmatis* in to the THP-1 cells, we anticipate that reducing macrophages uptake and multiplication of mycobacteria will be protective for the host. Therefore, the finding from this study suggest that conglutinin could be an important host defence collectin against tuberculosis infection.

The finding from this study needs to be further confirmed by repeating the experiments using *M. bovis* and bovine macrophages. If we found the effective inhibitory effect on the growth of *M. bovis* in vitro then we can further carry on the study by using animal models to find out the effect of rfBC to control bovine tuberculosis.

Over all, our finding suggested that conglutinin is involved in the host-pathogen interaction in tuberculosis infection by inhibiting the uptake of mycobacteria by macrophages by blocking the macrophage mannose and complement receptors. Conglutinin also help in the clearance of tuberculosis infection by preventing the mycobacterium immune evasion strategies. We also observed that conglutinin cause the down regulation of pro-inflammatory response against infection, which down regulate the cellular immune response against infection and prevent tissue damaging and also assist in the extracellular clearance of mycobacteria.

Therefore, findings from our study suggest that conglutinin plays an important host defense role against bovine tuberculosis infection by inhibiting phagocytosis of mycobacteria by macrophages via two key known mechanisms of interaction, i.e., the macrophage mannose receptor and complement iC3b receptors. Conglutinin probably enhances the clearance of mycobacterial infection by downplaying pathogen immune

evasion strategies and also augmenting the pro-inflammatory response against infection by down-regulating the cellular immune response against infection, suppressing tissue damage and steering toward the extracellular clearance of the pathogen. This study provides insights into previously unknown involvement of conglutinin and mycobacterial infection.

## **Chapter 7: References**

Abernethy, D.A., Upton, P., Higgins, I.M., McGrath, G., Goodchild, A.V., Rolfe, S.J., Broughan, J.M., Downs, S.H., Clifton-Hadley, R., Menzies, F.D., de la Rua-Domenech, R., Blissitt, M.J., Duignan, A. and More, S.J. (2013) 'Bovine tuberculosis trends in the UK and the Republic of Ireland, 1995-2010', *The Veterinary record*, 172(12), pp. 312.

Akira, S. (2011) 'Innate immunity and adjuvants', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 366(1579), pp. 2748-2755.

Aldwell, F., Wedlock, D. and Buddle, B. (1996) 'Bacterial metabolism, cytokine mRNA transcription and viability of bovine alveolar macrophages infected with *Mycobacterium bovis* BCG or virulent *M bovis*', *Immunology & Cell Biology*, 74(1), pp. 45-51.

Allen, A.R., Minozzi, G., Glass, E.J., Skuce, R.A., McDowell, S.W.J., Woolliams, J.A. and Bishop, S.C., 2010. Bovine tuberculosis: the genetic basis of host susceptibility. *Proceedings of the Royal Society B: Biological Sciences*, 277(1695), pp.2737-2745.

Andersen, O., Nielsen, E.H., Storgaard, P., Hojrup, P., Friis, P., Leslie, G. and Svehag, S. (1992) 'Biochemical and ultrastructural studies of the C-type lectin bovine conglutinin', *Journal of structural biology*, 109(3), pp. 201-207.

Andersen, O., SORENSEN, A., SVEHAG, S. and Fenouillet, E. (1991) 'Conglutinin Binds The HIV-1 Envelope Glycoprotein gp160 and Inhibits its Interaction with Cell Membrane CD4', *Scandinavian Journal of Immunology*, 33(1), pp. 81-88.

Ariki, S., Kojima, T., Gasa, S., Saito, A., Nishitani, C., Takahashi, M., Shimizu, T., Kurimura, Y., Sawada, N., Fujii, N. and Kuroki, Y. (2011) 'Pulmonary collectins play distinct roles in host defense against *Mycobacterium avium*', *Journal of immunology (Baltimore, Md.: 1950)*, 187(5), pp. 2586-2594.

Ayele, W., Neill, S., Zinsstag, J., Weiss, M. and Pavlik, I. (2004) 'Bovine tuberculosis: an old disease but a new threat to Africa', *The International Journal of Tuberculosis and Lung Disease*, 8(8), pp. 924-937.

Balseiro, A., González-Quirós, P., Rodríguez, Ó., Copano, M.F., Merediz, I., de Juan, L., Chambers, M.A., Delahay, R.J., Marreros, N. and Royo, L.J. (2013) 'Spatial relationships between Eurasian badgers (*Meles meles*) and cattle infected with *Mycobacterium bovis* in Northern Spain', *The Veterinary Journal*, 197(3), pp. 739-745.

Barry, C.E., Lee, R.E., Mdluli, K., Sampson, A.E., Schroeder, B.G., Slayden, R.A. and Yuan, Y., 1998. Mycolic acids: structure, biosynthesis and physiological functions. *Progress in lipid research*, 37(2), pp.143-179.

Barron, M., Tompkins, D., Ramsey, D. and Bosson, M. (2015) 'The role of multiple wildlife hosts in the persistence and spread of bovine tuberculosis in New Zealand', *New Zealand veterinary journal*, 63(sup1), pp. 68-76.

Bermingham, M.L., Bishop, S.C., Woolliams, J.A., Pong-Wong, R., Allen, A.R., McBride, S.H., Ryder, J.J., Wright, D.M., Skuce, R.A., McDowell, S.W. and Glass, E.J., 2014. Genome-wide association study identifies novel loci associated with resistance to bovine tuberculosis. *Heredity*, 112(5), p.543.

Bermingham, M.L., More, S.J., Good, M., Cromie, A.R., Higgins, I.M., Brotherstone, S. and Berry, D.P., 2009. Genetics of tuberculosis in Irish Holstein-Friesian dairy herds. *Journal of dairy science*, 92(7), pp.3447-3456.

Bobadilla, K., Sada, E., Jaime, M.E., González, Y., Ramachandra, L., Rojas, R.E., Pedraza-Sánchez, S., Michalak, C., González-Noriega, A. and Torres, M. (2013) 'Human phagosome processing of *Mycobacterium tuberculosis* antigens is modulated by interferon- $\gamma$  and interleukin-10', *Immunology*, 138(1), pp. 34-46.

Bordet, J. and Streng, O. (1909) *Les phénomènes d'adsorption et la congutinine du sérum de boeuf*.

Bourne, F.J., Donnelly, C.A., Cox, D.R., Gettinby, G., McInerney, J.P., Morrison, W.I. and Woodroffe, R. (2006) 'TB policy and the badger culling trials', *The Veterinary record*, 158(19), pp. 671-672.

Brotherstone, S., White, I.M.S., Coffey, M., Downs, S.H., Mitchell, A.P., Clifton-Hadley, R.S., More, S.J., Good, M. and Woolliams, J.A., 2010. Evidence of genetic resistance of cattle to infection with *Mycobacterium bovis*. *Journal of dairy science*, 93(3), pp.1234-1242.

Brummer, E. and Stevens, D.A. (2010) 'Collectins and fungal pathogens: roles of surfactant proteins and mannose binding lectin in host resistance', *Medical mycology*, 48(1), pp. 16-28.

Buccheri, S., Reljic, R., Caccamo, N., Ivanyi, J., Singh, M., Salerno, A. and Dieli, F. (2007) 'IL-4 depletion enhances host resistance and passive IgA protection against tuberculosis infection in BALB/c mice', *European journal of immunology*, 37(3), pp. 729-737.

Byrne, A.W., White, P.W., McGrath, G., James, O. and Martin, S.W. (2014) 'Risk of tuberculosis cattle herd breakdowns in Ireland: effects of badger culling effort, density and historic large-scale interventions', *Veterinary research*, 45(1), pp. 1.

Carroll, M.V., Lack, N., Sim, E., Krarup, A. and Sim, R.B. (2009) 'Multiple routes of complement activation by *Mycobacterium bovis* BCG', *Molecular immunology*, 46(16), pp. 3367-3378.

Cassidy, J. (2006) 'The pathogenesis and pathology of bovine tuberculosis with insights from studies of tuberculosis in humans and laboratory animal models', *Veterinary microbiology*, 112(2-4), pp. 151-161.

Cheeseman, C., Jones, G., Gallagher, J. and Mallinson, P. (1981) 'The population structure, density and prevalence of tuberculosis (*Mycobacterium bovis*) in badgers (*Meles meles*) from four areas in south-west England', *Journal of Applied Ecology*, , pp. 795-804.

Cleaveland, S., Shaw, D.J., Mfinanga, S.G., Shirima, G., Kazwala, R.R., Eblate, E. and Sharp, M. (2007) '*Mycobacterium bovis* in rural Tanzania: risk factors for infection in human and cattle populations', *Tuberculosis*, 87(1), pp. 30-43.



Cooper, A.M., Dalton, D.K., Stewart, T.A., Griffin, J.P., Russell, D.G. and Orme, I.M. (1993) 'Disseminated tuberculosis in interferon gamma gene-disrupted mice', *The Journal of experimental medicine*, 178(6), pp. 2243-2247.

Cooper, A.M., Magram, J., Ferrante, J. and Orme, I.M. (1997) 'Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis', *The Journal of experimental medicine*, 186(1), pp. 39-45.

Corner, L.A. (2006) 'The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk', *Veterinary microbiology*, 112(2), pp. 303-312.

Cosivi, O., Grange, J.M., Daborn, C.J., Raviglione, M.C., Fujikura, T., Cousins, D., Robinson, R.A., Huchzermeyer, H.F., de Kantor, I. and Meslin, F.X. (1998) 'Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries', *Emerging infectious diseases*, 4(1), pp. 59-70.

Cotter, T., Sheehan, S., Cryan, B., O'Shaughnessy, E., Cummins, H. and Bredin, C. (1996) 'Tuberculosis due to *Mycobacterium bovis* in humans in the south-west region of Ireland: is there a relationship with infection prevalence in cattle?', *Tubercle and Lung Disease*, 77(6), pp. 545-548.

Cousins, D. (2001) 'Mycobacterium bovis infection and control in domestic livestock', *Revue Scientifique Technique-Office International des Epizooties*, 20(1), pp. 71-85.

Cywes, C., Godenir, N.L., Hoppe, H.C., Scholle, R.R., Steyn, L.M., Kirsch, R.E. and Ehlers, M.R. (1996) 'Nonopsonic binding of *Mycobacterium tuberculosis* to human complement receptor type 3 expressed in Chinese hamster ovary cells', *Infect. Immun.*, 64, pp. 5373-5383.

Daffe, M. and Draper, P. (1997) 'The envelope layers of mycobacteria with reference to their pathogenicity', in *Advances in microbial physiology*. Elsevier, pp. 131-203.

Daffe, M. and Etienne, G. (1999) 'The capsule of *Mycobacterium tuberculosis* and its implications for pathogenicity', *Tubercle and Lung Disease*, 79(3), pp. 153-169.

Dankner, W.M., Waecker, N.J., Essey, M.A., Moser, K., Thompson, M. and Davis, C.E. (1993) 'Mycobacterium bovis infections in San Diego: a clinicoepidemiologic study of 73 patients and a historical review of a forgotten pathogen.', *Medicine*, 72(1), pp. 11-37.

Dannenbergh, A. (2001) 'Pathogenesis of pulmonary Mycobacterium bovis infection: basic principles established by the rabbit model', *Tuberculosis*, 81(1-2), pp. 87-96.

Dec, M. and Wernicki, A. (2006) 'Conglutinin, CL-43 and CL-46-three bovine collectins', *Pol J Vet Sci*, 9(4), pp. 265-275.

Dec, M., Wernicki, A., Puchalski, A., Urban-Chmiel, R. and Radej, S. (2012) 'Effect of conglutinin on phagocytic activity of bovine granulocytes', *Polish journal of veterinary sciences*, 15(3), pp. 455-462.

Defra (2014) 'The strategy for achieving officially bovine tuberculosis free status for England'.

Defra (2016) 'Monthly publication of official statistics on the incidence and prevalence of tuberculosis (TB) in cattle in Great Britain'.

Defra (2017) 'Quarterly statistics on tuberculosis (TB) in cattle in Great Britain'.

DEFRA, *Historical statistics notices on the incidence of tuberculosis (TB) in cattle in Great Britain, 2017*, F.a.R.A. Department of Environment, Editor. 2017, www.gov.uk.

Delahay, R., Smith, G., Barlow, A., Walker, N., Harris, A., Clifton-Hadley, R. and Cheeseman, C. (2007) 'Bovine tuberculosis infection in wild mammals in the South-West region of England: a survey of prevalence and a semi-quantitative assessment of the relative risks to cattle', *The Veterinary Journal*, 173(2), pp. 287-301.

De la Rúa-Domenech, R. (2006) 'Human Mycobacterium bovis infection in the United Kingdom: incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis', *Tuberculosis*, 86(2), pp. 77-109.

Demangel, C., Bertolino, P. and Britton, W.J. (2002) 'Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production', *European journal of immunology*, 32(4), pp. 994-1002.

Demetri, G.D. and Griffin, J.D. (1991) 'Granulocyte colony-stimulating factor and its receptor', *Blood*, 78(11), pp. 2791-2808.

Department for Environment, Food & Rural Affairs. The Government's Policy on Bovine TB and Badger Control in England; 2011. Available from: [https://http://www.gov.uk/government/uploads/system/uploads/attachment\\_data/file/69463/pb13691bovinetbpolicystatement.pdf](https://http://www.gov.uk/government/uploads/system/uploads/attachment_data/file/69463/pb13691bovinetbpolicystatement.pdf). Accessed Novemebr23, 2017.

Ding, A., Nathan, C.F., Graycar, J., Derynck, R., Stuehr, D.J. and Srimal, S. (1990) 'Macrophage deactivating factor and transforming growth factors-beta 1 -beta 2 and -beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma', *Journal of immunology (Baltimore, Md.: 1950)*, 145(3), pp. 940-944.

Domingo, M., Vidal, E. and Marco, A. (2014) 'Pathology of bovine tuberculosis', *Research in veterinary science*, 97, pp. S20-S29.

Donnelly, C.A., Woodroffe, R., Cox, D., Bourne, F.J., Cheeseman, C., Clifton-Hadley, R.S., Wei, G., Gettinby, G., Gilks, P. and Jenkins, H. (2006) 'Positive and negative effects of widespread badger culling on tuberculosis in cattle', *Nature*, 439(7078), pp. 843-846.

Driscoll, E.E., Hoffman, J.I., Green, L.E., Medley, G.F. and Amos, W., 2011. A preliminary study of genetic factors that influence susceptibility to bovine tuberculosis in the British cattle herd. *PLoS One*, 6(4), p.e18806.

Ehlers, S., Benini, J., Held, H.D., Roeck, C., Alber, G. and Uhlig, S. (2001) 'Alphabeta T cell receptor-positive cells and interferon-gamma, but not inducible nitric oxide synthase, are critical for granuloma necrosis in a mouse model of mycobacteria-induced pulmonary immunopathology', *The Journal of experimental medicine*, 194(12), pp. 1847-1859.

Ehlers, S. and Schaible, U.E. (2013) 'The granuloma in tuberculosis: dynamics of a host–pathogen collusion', *Frontiers in immunology*, 3, pp. 411.

Ehrlich, P. and Sachs, H. (1902) *Ueber den mechanismus der amboceptorenwirkung*. L. Schumacher.

Espitia, C., Vallecillo, A.J., Rodríguez, E., Echeverría-Valencia, G. and Ramón-Luing, L. (2012) *Host-pathogen interactions in tuberculosis*. INTECH Open Access Publisher.

Etter, E., Donado, P., Jori, F., Caron, A., Goutard, F. and Roger, F. (2006) 'Risk Analysis and Bovine Tuberculosis, a Re-emerging Zoonosis', *Annals of the New York Academy of Sciences*, 1081(1), pp. 61-73.

Eves, J. (1999) 'Impact of badger removal on bovine tuberculosis in east County Offaly', *Irish Veterinary Journal*, 52(4), pp. 199-203.

Feng, C.G., Kaviratne, M., Rothfuchs, A.G., Cheever, A., Hieny, S., Young, H.A., Wynn, T.A. and Sher, A. (2006) 'NK cell-derived IFN-gamma differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with *Mycobacterium tuberculosis*', *Journal of immunology (Baltimore, Md.: 1950)*, 177(10), pp. 7086-7093.

Ferguson, J.S., Voelker, D.R., McCormack, F.X. and Schlesinger, L.S. (1999) 'Surfactant protein D binds to *Mycobacterium tuberculosis* bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages', *Journal of immunology (Baltimore, Md.: 1950)*, 163(1), pp. 312-321.

Ferguson, J.S., Voelker, D.R., Ufnar, J.A., Dawson, A.J. and Schlesinger, L.S. (2002) 'Surfactant protein D inhibition of human macrophage uptake of *Mycobacterium tuberculosis* is independent of bacterial agglutination', *Journal of immunology (Baltimore, Md.: 1950)*, 168(3), pp. 1309-1314.

Ferguson, J.S., Weis, J.J., Martin, J.L. and Schlesinger, L.S. (2004) 'Complement protein C3 binding to *Mycobacterium tuberculosis* is initiated by the classical

pathway in human bronchoalveolar lavage fluid', *Infection and immunity*, 72(5), pp. 2564-2573.

Finlay, E.K., Berry, D.P., Wickham, B., Gormley, E.P. and Bradley, D.G., 2012. A genome wide association scan of bovine tuberculosis susceptibility in Holstein-Friesian dairy cattle. *PloS one*, 7(2), p.e30545.

Fitzgerald, S.D. and Kaneene, J.B. (2013) 'Wildlife reservoirs of bovine tuberculosis worldwide: hosts, pathology, surveillance, and control', *Veterinary pathology*, 50(3), pp. 488-499.

Flesch, I.E. and Kaufmann, S.H. (1990) 'Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor', *Infection and immunity*, 58(8), pp. 2675-2677.

Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A. and Bloom, B.R. (1993) 'An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection', *The Journal of experimental medicine*, 178(6), pp. 2249-2254.

Flynn, J.L., Goldstein, M.M., Chan, J., Triebold, K.J., Pfeffer, K., Lowenstein, C.J., Schreiber, R., Mak, T.W. and Bloom, B.R. (1995) 'Tumor necrosis factor- $\alpha$  is required in the protective immune response against Mycobacterium tuberculosis in mice', *Immunity*, 2(6), pp. 561-572.

Friis-Christiansen, P., Thiel, S., Svehag, S.-E., Dessau, R., Svendsen, P., Andersen, O., Laursen, S.B. and Jensenius, J.C. (1990) 'In vivo and in vitro antibacterial activity of conglutinin, a mammalian plasma lectin', *Scand. J. Immunol.*, 31, pp. 453-460.

Friis, P., Svehag, S.E., Andersen, O., Gahrn-Hansen, B. and Leslie, R.G. (1991) 'Conglutinin exhibits a complement-dependent enhancement of the respiratory burst of phagocytes stimulated by E. coli', *Immunology*, 74(4), pp. 680-684.

Gadjeva, M., Thiel, S. and Jensenius, J.C. (2001) 'The mannan-binding-lectin pathway of the innate immune response', *Current opinion in immunology*, 13(1), pp. 74-78.

Gallagher, J. and Clifton-Hadley, R. (2000) 'Tuberculosis in badgers; a review of the disease and its significance for other animals', *Research in veterinary science*, 69(3), pp. 203-217.

Gallagher, D.S., Ryan, A.M., Liou, L.S., Sastry, K.N. and Womack, J.E., 1993. Somatic cell mapping of conglutinin (CGN1) to cattle syntenic group U29 and fluorescence in situ localization to chromosome 28. *Mammalian Genome*, 4(12), pp.716-719.

Gallagher, J., Muirhead, R.H. and Burn, K.J. (1976) 'Tuberculosis in wild badgers (*Meles meles*) in Gloucestershire: pathology', *The Veterinary record*, 98(1), pp. 9-14.

Garnier, T., Eiglmeier, K., Camus, J.C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Doggett, J., Mayes, R., Keating, L., Wheeler, P.R., Parkhill, J., Barrell, B.G., Cole, S.T., Gordon, S.V. and Hewinson, R.G. (2003) 'The complete genome sequence of *Mycobacterium bovis*', *Proceedings of the National Academy of Sciences of the United States of America*, 100(13), pp. 7877-7882.

Gaynor, C.D., McCormack, F.X., Voelker, D.R., McGowan, S.E. and Schlesinger, L.S. (1995) 'Pulmonary surfactant protein A mediates enhanced phagocytosis of *Mycobacterium tuberculosis* by a direct interaction with human macrophages', *Journal of immunology (Baltimore, Md.: 1950)*, 155(11), pp. 5343-5351.

Gazzinelli, R.T., Oswald, I.P., James, S.L. and Sher, A. (1992) 'IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages', *Journal of immunology (Baltimore, Md.: 1950)*, 148(6), pp. 1792-1796.

Ghebrehiwet, B., Silvestri, L. and McDevitt, C. (1984) 'Identification of the Raji cell membrane-derived C1q inhibitor as a receptor for human C1q. Purification and immunochemical characterization', *The Journal of experimental medicine*, 160(5), pp. 1375-1389

Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., Julkunen, I. and Coccia, E.M. (2001) 'Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that

modulates T cell response', *Journal of immunology (Baltimore, Md.: 1950)*, 166(12), pp. 7033-7041.

Godiska, R., Chantry, D., Raport, C.J., Sozzani, S., Allavena, P., Leviten, D., Mantovani, A. and Gray, P.W. (1997) 'Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells', *The Journal of experimental medicine*, 185(9), pp. 1595-1604.

Gong, J.H., Zhang, M., Modlin, R.L., Linsley, P.S., Iyer, D., Lin, Y. and Barnes, P.F. (1996) 'Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression', *Infection and immunity*, 64(3), pp. 913-918.

Gormley, E., Doyle, M., Fitzsimons, T., McGill, K. and Collins, J. (2006) 'Diagnosis of Mycobacterium bovis infection in cattle by use of the gamma-interferon (Bovigam®) assay', *Veterinary microbiology*, 112(2), pp. 171-179.

Guglani, L. and Khader, S.A. (2010) 'Th17 cytokines in mucosal immunity and inflammation', *Current opinion in HIV and AIDS*, 5(2), pp. 120-127.

Guirado, E. and Schlesinger, L.S. (2013) 'Modeling the Mycobacterium tuberculosis Granuloma - the Critical Battlefield in Host Immunity and Disease', *Frontiers in immunology*, 4, pp. 98.

Haagsman, H.P., Hogenkamp, A., van Eijk, M. and Veldhuizen, E.J. (2008) 'Surfactant collectins and innate immunity', *Neonatology*, 93(4), pp. 288-294.

Hakansson, K. and Reid, K.B. (2000) 'Collectin structure: a review', *Protein Science*, 9(9), pp. 1607-1617.

Hansen, S., Holm, D., Moeller, V., Vitved, L., Bendixen, C., Reid, K.B., Skjoedt, K. and Holmskov, U. (2002) 'CL-46, a novel collectin highly expressed in bovine thymus and liver', *Journal of immunology (Baltimore, Md.: 1950)*, 169(10), pp. 5726-5734.

Hansen, S. and Holmskov, U. (2002) 'Lung surfactant protein D (SP-D) and the molecular diverted descendants: conglutinin, CL-43 and CL-46', *Immunobiology*, 205(4), pp. 498-517.

Hansen, S. and Holmskov, U. (1998) 'Structural aspects of collectins and receptors for collectins', *Immunobiology*, 199(2), pp. 165-189.

Hardie, R.M. and Watson, J.M. (1992) 'Mycobacterium bovis in England and Wales: past, present and future', *Epidemiology and infection*, 109(1), pp. 23-33.

Hartshorn, K.L., Sastry, K., Brown, D., White, M.R., Okarma, T.B., Lee, Y.M. and Tauber, A.I. (1993) 'Conglutinin acts as an opsonin for influenza A viruses', *Journal of immunology (Baltimore, Md.: 1950)*, 151(11), pp. 6265-6273.

Hernandez-Pando, R., Orozco, H., Sampieri, A., Pavon, L., Velasquillo, C., Larriva-Sahd, J., Alcocer, J.M. and Madrid, M.V. (1996) 'Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis', *Immunology*, 89(1), pp. 26-33.

Hetland, G. and Wiker, H.G. (1994) 'Antigen 85C on Mycobacterium bovis, BCG and M. tuberculosis promotes monocyte-CR3-mediated uptake of microbeads coated with mycobacterial products', *Immunology*, 82, pp. 445-449.

Hickling, T.P., Clark, H., Malhotra, R. and Sim, R.B. (2004) 'Collectins and their role in lung immunity', *Journal of leukocyte biology*, 75(1), pp. 27-33.

Hogan, L.H., Markofski, W., Bock, A., Barger, B., Morrissey, J.D. and Sandor, M. (2001) 'Mycobacterium bovis BCG-induced granuloma formation depends on gamma interferon and CD40 ligand but does not require CD28', *Infection and immunity*, 69(4), pp. 2596-2603.

Hogenkamp, A., Herias, M.V., Tooten, P.C., Veldhuizen, E.J. and Haagsman, H.P., 2007. Effects of surfactant protein D on growth, adhesion and epithelial invasion of intestinal Gram-negative bacteria. *Molecular immunology*, 44(14), pp.3517-3527.

Hogenkamp, A., van Eijk, M., van Dijk, A., van Asten, A.J., Veldhuizen, E.J. and Haagsman, H.P. (2006) 'Characterization and expression sites of newly identified chicken collectins', *Molecular immunology*, 43(10), pp. 1604-1616.



Hoheisel, G., Izbicki, G., Roth, M., Chan, C., Leung, J., Reichenberger, F., Schauer, J. and Perruchoud, A. (1998) 'Compartmentalization of pro-inflammatory cytokines in tuberculous pleurisy', *Respiratory medicine*, 92(1), pp. 14-17.

Holmskov, U.L. (2000) 'Collectins and collectin receptors in innate immunity', *Apmis*, 108(S100), pp. 7-59.

Holmskov, U., Jensenius, J.C., Tornøe, I. and Lovendahl, P. (1998) 'The plasma levels of conglutinin are heritable in cattle and low levels predispose to infection', *Immunology*, 93(3), pp. 431-436.

Holmskov, U., Lawson, P., Teisner, B., Tornøe, I., Willis, A.C., Morgan, C., Koch, C. and Reid, K.B. (1997) 'Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule', *The Journal of biological chemistry*, 272(21), pp. 13743-13749.

Holmskov, U., Teisner, B., Pedersen, N.T., Laursen, S.B., Rasmussen, H.B. and Jensenius, J.C. (1992) 'Tissue localization of conglutinin, a bovine C-type lectin', *Immunology*, 76(1), pp. 169-173.

Hoppe, H. and Reid, K. (1994) 'Collectins—soluble proteins containing collagenous regions and lectin domains—and their roles in innate immunity', *Protein Science*, 3(8), pp. 1143-1158.

Hu, B. and Yasui, K. (1997) 'Effects of colony-stimulating factors (CSFs) on neutrophil apoptosis: possible roles at inflammation site', *International journal of hematology*, 66(2), pp. 179-188.

Humblet, M., Boschioli, M.L. and Saegerman, C. (2009) 'Classification of worldwide bovine tuberculosis risk factors in cattle: a stratified approach', *Veterinary research*, 40(5), pp. 1-24.

Ingram, D. and Barnum, D. (1965) 'Fluctuations of the Level of Conglutinin in Bovine Sera', in *Blood Groups of Animals*. Springer, pp. 99-103.

Ingram, D. and Mitchell, W. (1970) 'Conglutinin levels in dairy cattle: changes associated with parturition.', *American Journal of Veterinary Research*, 31, pp. 487-492.

INGRAM, D.G. (1959) 'The conglutination phenomenon. XIII. In vivo interactions of conglutinin and experimental bacterial infection', *Immunology*, 2, pp. 322-333.

Jankute, M., Grover, S., Birch, H.L. and Besra, G.S., 2014. Genetics of mycobacterial arabinogalactan and lipoarabinomannan assembly. In *Molecular Genetics of Mycobacteria, Second Edition* (pp. 535-557). American Society of Microbiology.

Juffermans, N.P., Verbon, A., Belisle, J.T., Hill, P.J., Speelman, P., van DEVENTER, S.J. and van der Poll, T. (2000) 'Mycobacterial lipoarabinomannan induces an inflammatory response in the mouse lung: a role for interleukin-1', *American journal of respiratory and critical care medicine*, 162(2), pp. 486-489.

Jungblut, P.R., Schaible, U.E., Mollenkopf, H.J., Zimny-Arndt, U., Raupach, B., Mattow, J., Halada, P., Lamer, S., Hagens, K. and Kaufmann, S.H.E., 1999. Comparative proteome analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains: towards functional genomics of microbial pathogens. *Molecular microbiology*, 33(6), pp.1103-1117.

Junqueira-Kipnis, A.P., Kipnis, A., Jamieson, A., Juarrero, M.G., Diefenbach, A., Rautlet, D.H., Turner, J. and Orme, I.M. (2003) 'NK cells respond to pulmonary infection with Mycobacterium tuberculosis, but play a minimal role in protection', *Journal of immunology (Baltimore, Md.: 1950)*, 171(11), pp. 6039-6045.

Kadarmideen, H.N., Ali, A.A., Thomson, P.C., Müller, B. and Zinsstag, J., 2011. Polymorphisms of the SLC11A1 gene and resistance to bovine tuberculosis in African Zebu cattle. *Animal genetics*, 42(6), pp.656-658.

Kanakaraj, P., Ngo, K., Wu, Y., Angulo, A., Ghazal, P., Harris, C.A., Siekierka, J.J., Peterson, P.A. and Fung-Leung, W.P. (1999) 'Defective interleukin (IL)-18-mediated natural killer and T helper cell type 1 responses in IL-1 receptor-associated kinase (IRAK)-deficient mice', *The Journal of experimental medicine*, 189(7), pp. 1129-1138.

Kawai, T., Kase, T., Suzuki, Y., Eda, S., Sakamoto, T., Ohtani, K. and Wakamiya, N. (2007) 'Anti-influenza A virus activities of mannan-binding lectins and bovine conglutinin', *Journal of Veterinary Medical Science*, 69(2), pp. 221-224.

Kean, J., Barlow, N. and Hickling, G. (1999) 'Evaluating potential sources of bovine tuberculosis infection in a New Zealand cattle herd', *New Zealand Journal of Agricultural Research*, 42(1), pp. 101-106.

Kishore, U., Bernal, A.L., Kamran, M.F., Saxena, S., Singh, M., Sarma, P.U., Madan, T. and Chakraborty, T. (2005) 'Surfactant proteins SP-A and SP-D in human health and disease', *ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS-ENGLISH EDITION*-, 53(5), pp. 399.

Kishore, U., Greenhough, T.J., Waters, P., Shrive, A.K., Ghai, R., Kamran, M.F., Bernal, A.L., Reid, K.B., Madan, T. and Chakraborty, T. (2006) 'Surfactant proteins SP-A and SP-D: structure, function and receptors', *Molecular immunology*, 43(9), pp. 1293-1315.

Kuroki, Y., Takahashi, M. and Nishitani, C. (2007) 'Pulmonary collectins in innate immunity of the lung', *Cellular microbiology*, 9(8), pp. 1871-1879.

Lachmann, P. and Hughes-Jones, N. (1984) 'Initiation of complement activation', *Springer seminars in immunopathology*. Springer, 143-162.

LACHMANN, P.J. (1962) 'A comparison of some properties of bovine conglutinin with those of rabbit immuno-conglutinin', *Immunology*, 5, pp. 687-705.

Lachmann, P.J. and Muller-Eberhard, H.J. (1968) 'The demonstration in human serum of "conglutinin-activating factor" and its effect on the third component of complement', *Journal of immunology (Baltimore, Md.: 1950)*, 100(4), pp. 691-698.

Laursen, S.B., Thiel, S., Teisner, B., Holmskov, U., Wang, Y., Sim, R.B. and Jensenius, J.C. (1994) 'Bovine conglutinin binds to an oligosaccharide determinant presented by iC3b, but not by C3, C3b or C3c', *Immunology*, 81(4), pp. 648-654.

Leal, I.S., Smedegård, B., Andersen, P. and Appelberg, R. (1999) 'Interleukin-6 and Interleukin-12 Participate in Induction of a Type 1 Protective T-Cell Response during Vaccination with a Tuberculosis Subunit Vaccine', *Infection and immunity*, 67(11), pp. 5747-5754.

Lee, Y.M., Leiby, K.R., Allar, J., Paris, K., Lerch, B. and Okarma, T.B. (1991) 'Primary structure of bovine conglutinin, a member of the C-type animal lectin family', *The Journal of biological chemistry*, 266(5), pp. 2715-2723.

Lenzini, L., Rottoli, P. and Rottoli, L. (1977) 'The spectrum of human tuberculosis', *Clinical and experimental immunology*, 27(2), pp. 230-237.

Leon, M.A. (1957) 'Role of Cations in Conglutination and in Formation of Properdin Zymosan Complex from Bovine Serum', *Proceedings of the Society for Experimental Biology and Medicine*, 96(1), pp. 202-204.

Lepper, A., Corner, L. and Pearson, W., 1977. Serological responses in experimental bovine tuberculosis. *Australian veterinary journal*, 53(7), pp.301-305.

Lim, A., Steibel, J.P., Coussens, P.M., Grooms, D.L. and Bolin, S.R. (2012) 'Differential gene expression segregates cattle confirmed positive for bovine tuberculosis from antemortem tuberculosis test-false positive cattle originating from herds free of bovine tuberculosis', *Veterinary medicine international*, 2012.

Liou, L.S., Sastry, R., Hartshorn, K.L., Lee, Y.M., Okarma, T.B., Tauber, A.I. and Sastry, K.N. (1994) 'Bovine conglutinin (BC) mRNA expressed in liver: cloning and characterization of the BC cDNA reveals strong homology to surfactant protein-D', *Gene*, 141(2), pp. 277-281

Loveless, R.W., Holmskov, U. and Feizi, T. (1995) 'Collectin-43 is a serum lectin with a distinct pattern of carbohydrate recognition', *Immunology*, 85(4), pp. 651-659.

Lucey, D.R., Clerici, M. and Shearer, G.M. (1996) 'Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases', *Clinical microbiology reviews*, 9(4), pp. 532-562.

LU, J., WIEDEMANN, H., HOLMSKOV, U., THIEL, S., TIMPL, R. and REID, K. (1993) 'Structural similarity between lung surfactant protein D and conglutinin', *The FEBS Journal*, 215(3), pp. 793-799.

Malhotra, R., Laursen, S.B., Willis, A.C. and Sim, R.B. (1993) 'Localization of the receptor-binding site in the collectin family of proteins', *The Biochemical journal*, 293 ( Pt 1)(Pt 1), pp. 15-19.

Malhotra, R., Thiel, S., Reid, K.B. and Sim, R.B. (1990) 'Human leukocyte C1q receptor binds other soluble proteins with collagen domains', *The Journal of experimental medicine*, 172(3), pp. 955-959.

Martinez, F.O., Sica, A., Mantovani, A. and Locati, M. (2008) 'Macrophage activation and polarization', *Frontiers in bioscience : a journal and virtual library*, 13, pp. 453-461.

Matsushita, M., Endo, Y. and Fujita, T. (2000) 'Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease', *Journal of immunology (Baltimore, Md.: 1950)*, 164(5), pp. 2281-2284.

Matsushita, M. and Fujita, T. (1992) 'Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease', *The Journal of experimental medicine*, 176(6), pp. 1497-1502.

Mayer-Barber, K.D., Barber, D.L., Shenderov, K., White, S.D., Wilson, M.S., Cheever, A., Kugler, D., Hieny, S., Caspar, P., Núñez, G., Schlueter, D., Flavell, R.A., Sutterwala, F.S. and Sher, A. (2010) 'Cutting edge: Caspase-1 independent IL-1 $\beta$  production is critical for host resistance to *Mycobacterium tuberculosis* and does not require TLR signaling in vivo', *Journal of Immunology*, 184(7), pp. 3326-3330.

Menzies, F. and Neill, S. (2000) 'Cattle-to-cattle transmission of bovine tuberculosis', *The Veterinary Journal*, 160(2), pp. 92-106.

Michel, A.L., Müller, B. and Van Helden, P.D. (2010) 'Mycobacterium bovis at the animal-human interface: A problem, or not?', *Veterinary microbiology*, 140(3), pp. 371-381.

Mohan, V.P., Scanga, C.A., Yu, K., Scott, H.M., Tanaka, K.E., Tsang, E., Tsai, M.M., Flynn, J.L. and Chan, J. (2001) 'Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology', *Infection and immunity*, 69(3), pp. 1847-1855.

More, S.J. and Good, M. (2006) 'The tuberculosis eradication programme in Ireland: a review of scientific and policy advances since 1988', *Veterinary microbiology*, 112(2), pp. 239-251.

Mostowy, S., Inwald, J., Gordon, S., Martin, C., Warren, R., Kremer, K., Cousins, D. and Behr, M.A. (2005) 'Revisiting the evolution of *Mycobacterium bovis*', *Journal of Bacteriology*, 187(18), pp. 6386-6395.

Muller, B., Durr, S., Alonso, S., Hattendorf, J., Laise, C.J., Parsons, S.D., van Helden, P.D. and Zinsstag, J. (2013) 'Zoonotic *Mycobacterium bovis*-induced tuberculosis in humans', *Emerging infectious diseases*, 19(6), pp. 899-908.

Munder, M., Mallo, M., Eichmann, K. and Modolell, M. (2001) 'Direct stimulation of macrophages by IL-12 and IL-18—a bridge built on solid ground', *Immunology letters*, 75(2), pp. 159-160.

Murhead, R.H. and Burns, K.J. (1974) 'Tuberculosis in wild badgers in Gloucestershire: epidemiology', *The Veterinary record*, 95(24), pp. 552-555.

Nair, S.V., Pearce, S., Green, P.L., Mahajan, D., Newton, R.A. and Raftos, D.A. (2000) 'A collectin-like protein from tunicates', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 125(2), pp. 279-289.

Nakagawa, H., Komorita, N., Shibata, F., Ikesue, A., Konishi, K., Fujioka, M. and Kato, H. (1994) 'Identification of cytokine-induced neutrophil chemoattractants (CINC), rat GRO/CINC-2 alpha and CINC-2 beta, produced by granulation tissue in culture: purification, complete amino acid sequences and characterization', *The Biochemical journal*, 301 ( Pt 2)(Pt 2), pp. 545-550.

Nakao, M., Kajiya, T., Sato, Y., Somamoto, T., Kato-Unoki, Y., Matsushita, M., Nakata, M., Fujita, T. and Yano, T. (2006) 'Lectin pathway of bony fish complement:

identification of two homologs of the mannose-binding lectin associated with MASP2 in the common carp (*Cyprinus carpio*)', *Journal of immunology (Baltimore, Md.: 1950)*, 177(8), pp. 5471-5479.

Neill, S., Bryson, D. and Pollock, J. (2001) 'Pathogenesis of tuberculosis in cattle', *Tuberculosis*, 81(1-2), pp. 79-86.

Ni Bhuachalla, D., Corner, L.A., More, S.J. and Gormley, E. (2014) 'The role of badgers in the epidemiology of *Mycobacterium bovis* infection (tuberculosis) in cattle in the United Kingdom and the Republic of Ireland: current perspectives on control strategies',.

Olea-Popelka, F., Costello, E., White, P., McGrath, G., Collins, J., O'Keeffe, J., Kelton, D., Berke, O., More, S. and Martin, S. (2008) 'Risk factors for disclosure of additional tuberculous cattle in attested-clear herds that had one animal with a confirmed lesion of tuberculosis at slaughter during 2003 in Ireland', *Preventive veterinary medicine*, 85(1), pp. 81-91.

O'Leary, S., O'Sullivan, M.P. and Keane, J. (2011) 'IL-10 blocks phagosome maturation in *Mycobacterium tuberculosis*-infected human macrophages', *American Journal of Respiratory Cell and Molecular Biology*, 45(1), pp. 172-180.

Pangburn, M.K. and Muller-Eberhard, H.J. (1984) 'The alternative pathway of complement', *Springer seminars in immunopathology*. Springer, 163-192.

Payne, A., Boschioli, M.L., Gueneau, E., Moyen, J.L., Rambaud, T., Dufour, B., Gilot-Fromont, E. and Hars, J. (2013) 'Bovine tuberculosis in "Eurasian" badgers (*Meles meles*) in France', *European journal of wildlife research*, 59(3), pp. 331-339.

Phillips, C., Foster, C., Morris, P. and Teverson, R. (2003) 'The transmission of *Mycobacterium bovis* infection to cattle', *Research in veterinary science*, 74(1), pp. 1-15.

Pollock, J. and Neill, S. (2002) '*Mycobacterium bovis* infection and tuberculosis in cattle', *The Veterinary Journal*, 163(2), pp. 115-127.

Pollock, J., Pollock, D., Campbell, D., Girvin, R., Crockard, A., Neill, S. and Mackie, D. (1996) 'Dynamic changes in circulating and antigen-responsive T-cell subpopulations post-*Mycobacterium bovis* infection in cattle', *Immunology*, 87(2), pp. 236-241.

Public Health England (2015) 'Mycobacterium bovis TB case notifications by country, UK, 1999-2014.

Ramos, D., Silva, P. and Dellagostin, O. (2015) 'Diagnosis of bovine tuberculosis: review of main techniques', *Brazilian Journal of Biology*, 75(4), pp. 830-837.

Ramanathan, V.D., Curtis, J. and Turk, J.L. (1980) 'Activation of the alternative pathway of complement by mycobacteria and cord factor', *Infect. Immun.*, 29, pp. 30-35.

Reading, P.C., Holmskov, U. and Anders, E.M. (1998) 'Antiviral activity of bovine collectins against rotaviruses.', *Journal of General Virology*, 79(9), pp. 2255-2263.

Reading, P.C., Morey, L.S., Crouch, E.C. and Anders, E.M., 1997. Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection of mice. *Journal of virology*, 71(11), pp.8204-8212.

Ritacco, V., Lopez, B., De Kantor, I., Barrera, L., Errico, F. and Nader, A. (1991) 'Reciprocal cellular and humoral immune responses in bovine tuberculosis', *Research in veterinary science*, 50(3), pp. 365-367.

Rose, C.E., Sung, S.J. and Fu, S.M. (2003) 'Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung', *Microcirculation*, 10(3-4), pp. 273-288.

ROSS, G.D. (1986) 'Opsonization and membrane complement receptors', in *Immunobiology of the Complement System*. Elsevier, pp. 87-114.

Roy, C.J. and Milton, D.K. (2004) *Airborne transmission of communicable infection-the elusive pathway*,

Ruhwald, M., Bjerregaard-Andersen, M., Rabna, P., Eugen-Olsen, J. and Ravn, P. (2009) 'IP-10, MCP-1, MCP-2, MCP-3, and IL-1RA hold promise as biomarkers for



infection with *M. tuberculosis* in a whole blood based T-cell assay', *BMC research notes*, 2(1), pp. 19.

Ruscetti, F., Varesio, L., Ochoa, A. and Ortaldo, J. (1993) 'Pleiotropic Effects of Transforming Growth Factor- $\beta$  on Cells of the Immune System', *Annals of the New York Academy of Sciences*, 685(1), pp. 488-500.

Russell, D.G., Barry, C.E., 3rd and Flynn, J.L. (2010) 'Tuberculosis: what we don't know can, and does, hurt us', *Science (New York, N.Y.)*, 328(5980), pp. 852-856.

Sano, H. and Kuroki, Y. (2005) 'The lung collectins, SP-A and SP-D, modulate pulmonary innate immunity', *Molecular immunology*, 42(3), pp. 279-287.

Schiller, I., Oesch, B., Vordermeier, H., Palmer, M., Harris, B., Orloski, K., Buddle, B., Thacker, T., Lyashchenko, K. and Waters, W. (2010) 'Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication', *Transboundary and emerging diseases*, 57(4), pp. 205-220.

Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S.C. and Dinarello, C.A. (1990) 'Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF', *Blood*, 75(1), pp. 40-47.

Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R. and Horwitz, M.A. (1990) 'Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3', *Journal of immunology (Baltimore, Md.: 1950)*, 144(7), pp. 2771-2780

Schlesinger, L.S. and Horwitz, M.A. (1994) 'A role for natural antibody in the pathogenesis of leprosy: antibody in nonimmune serum mediates C3 fixation to the *Mycobacterium leprae* surface and hence phagocytosis by human mononuclear phagocytes', *Infect. Immun.*, 62, pp. 280-289.

Schmiedel, A. (1968) 'Development and present state of bovine tuberculosis in man', *Bulletin of the International Union against Tuberculosis*, 40, pp. 5-32.

Schneider, A., Kruger, C., Steigleder, T., Weber, D., Pitzer, C., Laage, R., Aronowski, J., Maurer, M.H., Gassler, N., Mier, W., Hasselblatt, M., Kollmar, R., Schwab, S., Sommer, C., Bach, A., Kuhn, H.G. and Schabitz, W.R. (2005) 'The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis', *The Journal of clinical investigation*, 115(8), pp. 2083-2098.

Seth, M., Lamont, E.A., Janagama, H.K., Widdel, A., Vulchanova, L., Stabel, J.R., Waters, W.R., Palmer, M.V. and Sreevatsan, S. (2009) 'Biomarker discovery in subclinical mycobacterial infections of cattle', *PLoS One*, 4(5), pp. e5478.

Shaw, T.C., Thomas, L.H. and Friedland, J.S. (2000) 'Regulation of IL-10 secretion after phagocytosis of Mycobacterium tuberculosis by human monocytic cells', *Cytokine*, 12(5), pp. 483-486.

Sim, R., Day, A., Moffatt, B. and Fontaine, M. (1993) '[1] Complement factor I and cofactors in control of complement system convertase enzymes', *Methods in enzymology*, 223, pp. 13-35.

Skinner, M.A., Ramsay, A., Buchan, G., Keen, D., Ranasinghe, C., Slobbe, L., Collins, D., De Lisle, G. and Buddle, B. (2003) 'A DNA prime-live vaccine boost strategy in mice can augment IFN- $\gamma$  responses to mycobacterial antigens but does not increase the protective efficacy of two attenuated strains of Mycobacterium bovis against bovine tuberculosis', *Immunology*, 108(4), pp. 548-555.

Skuce, R.A., Allen, A.R. and McDowell, S.W. (2012) 'Herd-level risk factors for bovine tuberculosis: a literature review', *Veterinary medicine international*, 2012.

Skuce, R.A., Allen, A.R., McDowell, S.W. and Branch, B. (2011) 'Bovine tuberculosis (TB): a review of cattle-to-cattle transmission, risk factors and susceptibility', *Bacteriology Branch Veterinary Sciences Division Agrifood and Biosciences Institute, October*,.

Sliwa-Dominiak, J., Tokarz-Deptuła, B., Niedźwiedzka-Rystwej, P. and Deptuła, W. (2010) 'Conglutinin-an important element of natural immunity in ruminants (a review)', *Acta Veterinaria Brno*, 79(1), pp. 99-104.

Smith, D., Hänisch, H., Bancroft, G. and Ehlers, S. (1997) 'T-cell-independent granuloma formation in response to *Mycobacterium avium*: role of tumour necrosis factor- $\alpha$  and interferon- $\gamma$ ', *Immunology*, 92(4), pp. 413-421.

Smith, G.C., Delahay, R.J., McDonald, R.A. and Budgey, R. (2016) 'Model of Selective and Non-Selective Management of Badgers (*Meles meles*) to Control Bovine Tuberculosis in Badgers and Cattle', *PloS one*, 11(11), pp. e0167206.

Smith, N.H., Gordon, S.V., de la Rua-Domenech, R., Clifton-Hadley, R.S. and Hewinson, R.G. (2006) 'Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*', *Nature Reviews Microbiology*, 4(9), pp. 670-681.

Srivastava, K., Chauhan, D.S., Gupta, P., Singh, H.B., Sharma, V.D., Yadav, V., Thakral, S.S., Dharamdheeran, J.S., Nigam, P., Prasad, H.K. and Katoch, V.M., 2008. Isolation of *Mycobacterium bovis* & *M. tuberculosis* from cattle of some farms in north India-possible relevance in human health. *Indian Journal of Medical Research*, 128(1), p.26.

Sun, L., Song, Y., Riaz, H., Yang, H., Hua, G., Guo, A. and Yang, L., 2012. Polymorphisms in toll-like receptor 1 and 9 genes and their association with tuberculosis susceptibility in Chinese Holstein cattle. *Veterinary immunology and immunopathology*, 147(3-4), pp.195-201.

Sundaramurthy, V. and Pieters, J. (2007) 'Interactions of pathogenic mycobacteria with host macrophages', *Microbes and Infection*, 9(14), pp. 1671-1679.

Swift, B.M., Convery, T.W. and Rees, C.E., 2016. Evidence of *Mycobacterium tuberculosis* complex bacteraemia in intradermal skin test positive cattle detected using phage-RPA. *Virulence*, 7(7), pp.779-788.

Tebug, S., Njunga, G.R., Chagunda, M.G., Mapemba, J.P., Awah-Ndukum, J. and Wiedemann, S. (2014) 'Risk, knowledge and preventive measures of smallholder dairy farmers in northern Malawi with regard to zoonotic brucellosis and bovine tuberculosis', *Onderstepoort Journal of Veterinary Research*, 81(1), pp. 01-06.

Tenner, A.J. (1993) 'Functional aspects of the C1q receptors', *Behring Institute Mitteilungen*, (93)(93), pp. 241-253.

Teixeira, J.E., Heron, B.T. and Huston, C.D. (2008) 'C1q-and collectin-dependent phagocytosis of apoptotic host cells by the intestinal protozoan *Entamoeba histolytica*', *The Journal of infectious diseases*, 198(7), pp. 1062-1070.

Thacker, T.C., Palmer, M.V. and Waters, W.R. (2007) 'Associations between cytokine gene expression and pathology in *Mycobacterium bovis* infected cattle', *Veterinary immunology and immunopathology*, 119(3), pp. 204-213.

Thoen, C.O., Steele, J.H. and Gilsdorf, M.J. (2008) *Mycobacterium bovis infection in animals and humans*. John Wiley & Sons.

Tian, T., Woodworth, J., Skold, M. and Behar, S.M. (2005) 'In vivo depletion of CD11c+ cells delays the CD4+ T cell response to *Mycobacterium tuberculosis* and exacerbates the outcome of infection', *Journal of immunology (Baltimore, Md.: 1950)*, 175(5), pp. 3268-3272.

Torgerson, P.R. and Torgerson, D.J. (2010) 'Public health and bovine tuberculosis: what's all the fuss about?', *Trends in microbiology*, 18(2), pp. 67-72.

Toossi, Z. and Ellner, J.J. (1998) 'The role of TGF $\beta$  in the pathogenesis of human tuberculosis', *Clinical immunology and immunopathology*, 87(2), pp. 107-114.

U.S. Department of Agriculture. 2005. Bovine tuberculosis eradication: uniform methods and rules, effective January 1, 2005; USDA-APHIS 91-45-011. Available at: <http://www.aphis.usda.gov/vs/nahps/tb/tb-umr.pdf>, accessed 20/12/2017

van Crevel, R., Karyadi, E., Preyers, F., Leenders, M., Kullberg, B., Nelwan, R.H. and van der Meer, Jos WM (2000) 'Increased production of interleukin 4 by CD4 and CD8 T cells from patients with tuberculosis is related to the presence of pulmonary cavities', *The Journal of infectious diseases*, 181(3), pp. 1194-1197.

Van de Wetering, J Koenraad, van Golde, L.M. and Batenburg, J.J. (2004) 'Collectins', *European Journal of Biochemistry*, 271(7), pp. 1229-1249.

Vordermeier, M., Gordon, S.V. and Hewinson, R.G. (2011) 'Mycobacterium bovis antigens for the differential diagnosis of vaccinated and infected cattle', *Veterinary microbiology*, 151(1-2), pp. 8-13.

Wang, J., Kishore, U. and Reid, K. (1995) 'A Recombinant polypeptide, composed of the  $\alpha$ -helical neck region and the carbohydrate recognition domain of conglutinin, self-associates to give a functionally intact homotrimer', *FEBS letters*, 376(1-2), pp. 6-10.

Waters, W.R., Palmer, M.V., Buddle, B.M. and Vordermeier, H.M. (2012) 'Bovine tuberculosis vaccine research: historical perspectives and recent advances', *Vaccine*, 30(16), pp. 2611-2622.

Weaver, T.E. and Whitsett, J.A. (1991) 'Function and regulation of expression of pulmonary surfactant-associated proteins', *The Biochemical journal*, 273(Pt 2)(Pt 2), pp. 249-264.

Weikert, L.F., Edwards, K., Chronos, Z.C., Hager, C., Hoffman, L. and Shepherd, V.L. (1997) 'SP-A enhances uptake of bacillus Calmette-Guerin by macrophages through a specific SP-A receptor', *The American Journal of Physiology*, 272(5 Pt 1), pp. L989-95.

Whipple, D.L., Bolin, C.A. and Miller, J.M. (1996) 'Distribution of lesions in cattle infected with Mycobacterium bovis', *Journal of Veterinary Diagnostic Investigation*, 8(3), pp. 351-354.

Willment, J.A. and Brown, G.D. (2008) 'C-type lectin receptors in antifungal immunity', *Trends in microbiology*, 16(1), pp. 27-32.

Wolf, A.J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K. and Ernst, J.D. (2008) 'Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs', *The Journal of experimental medicine*, 205(1), pp. 105-115.

Wood, P., Corner, L., Rothel, J., Ripper, J., Fifis, T., McCormick, B., Francis, B., Melville, L., Small, K. and De Witte, K. (1992) 'A field evaluation of serological and

cellular diagnostic tests for bovine tuberculosis', *Veterinary microbiology*, 31(1), pp. 71-79.

World Organisation for World Organisation for Animal Health available from [https://www.oie.int/wahis\\_2/public/wahid.php/Diseaseinformation/statusdetail](https://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail). Accessed March 2018.

Wu, H., Kuzmenko, A., Wan, S., Schaffer, L., Weiss, A., Fisher, J.H., Kim, K.S. and McCormack, F.X. (2003) 'Surfactant proteins A and D inhibit the growth of Gram-negative bacteria by increasing membrane permeability', *The Journal of clinical investigation*, 111(10), pp. 1589-1602.

Yoneda, T. and Ellner, J.J. (1998) 'CD4 T cell and natural killer cell-dependent killing of *Mycobacterium tuberculosis* by human monocytes', *American journal of respiratory and critical care medicine*, 158(2), pp. 395-403.