Development and Optimization of Real-Time PCR Assays to Detect Anti-Microbial Immune Factors and their Response to Type I and II Interferons

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Submitted in fulfillment of the requirements for the degree of

Masters of Medical Science in Immunology in the School of Laboratory Medicine and Medical Sciences

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&

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27th January 2016

Table of Contents

Table of Contents i

Preface iii

Abstract iii

Acknowledgements ii

Declaration iii

List of Figures iv

List of Tables vi

List of Appendixes vi

Abbreviations vi

1. CHAPTER ONE: Introduction and Literature Review 8

1.1 Human Immunodeficiency Virus (HIV) 8

1.2 Tuberculosis (TB) 6

1.3 TB/HIV Co-infection 9

1.4 Cytokines are associated with HIV infection. 11

1.4.1 Interferon gamma (IFN-γ) 12

1.4.2 Interferon alpha (IFNα2a) 13

1.4.3 The Complex Interplay of Type I/II Interferon 15

1.5 Toll-like Receptors (TLRs) 15

1.6 Monocytes, Macrophages and the Innate Immune System 17

1.6.1 Challenges in TB vaccine design and the need to understand mechanisms of innate anti-TB immunity 17

1.6.2 Vitamin-D Dependant Antimicrobial Peptides and their Role in Innate Immune Defence 19

1.6.3 Innate Protection: Vitamin D Dependent Antimicrobial Pathway 22

1.7 Aim of Study 25

1.8 Hypothesis 26

1.9 Rationale 27

1.10 Specific Objectives 27

2. CHAPTER TWO: Optimisation of qPCR Assays for Reliable Detection of Transcripts of Interest in the Vitamin-D Dependant Antimicrobial Pathway in the THP-1 Cell Line 28

2.1 Brief Introduction 28

2.1.1 Traditional PCR vs. Real-Time PCR 28

2.1.2 Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines 30

2.1.3 Optimisation of Issues Encountered in Real-Time PCR 33

2.2 Methods and Materials ... 36
2.2.1 Cells and Reagents ... 36
2.2.2 RNA Isolation and cDNA Synthesis ... 37
2.2.3 Primers and Real Time PCR ... 39
2.3 Results ... 40
2.3.1 Bioinformatic Validation of Primers ... 40
2.3.1 Validation of Genes of Interest by Agarose Gel Electrophoresis ... 44
2.3.2 Optimisation of qPCR Conditions to Remove Primer-Dimer ... 44
2.3.3 Optimisation of qPCR Conditions to Remove Non-Specific Amplification ... 45
2.3.4 Assessment of RNA Quality ... 48
2.3.5 Formation of Standard Curves for Relative Analyses ... 49
2.3.6 Lack of Reliable Expression of DEFB4 in THP-1 Cells ... 50
2.3.7 Validation of Reference Genes ... 52
2.4 Discussion and Conclusion ... 53
3 CHAPTER THREE: Effect of Type I/II Interferon Stimulation on the Expression of Transcripts of Interest in Healthy Patient CD14+ Cells and the THP-1 Monocytic Cell Line ... 55
3.1 Brief Introduction ... 55
3.1 Methods and Materials ... 59
3.1.1 Cells and Reagents ... 59
3.1.2 RNA Isolation and cDNA Synthesis ... 60
3.1.3 Primers and Real Time PCR ... 61
3.1.4 Analysis of Gene Expression ... 61
3.2 Results ... 62
3.2.1 Comparison of basal expression level for transcripts of interest in THP-1 cells and primary monocytes ... 62
3.2.2 Impact of interferon stimulations on gene expression in THP-1 cells ... 64
3.2.3 Impact of interferon stimulations on gene expression in primary CD14+ monocytes ... 66
3.3 Conclusions and Discussion ... 66
3.3.1 Expression of the pathway of interest differed in THP-1 cells and primary CD14+ monocytes 67
3.3.2 Interferon gamma upregulated key immune factors in CD14+ monocytes and unexpectedly down-regulated some of the same factors in THP-1 cells ... 68
3.3.3 Interferon Alpha 2a stimulation did not significantly modulate the expression of key immune factors in CD14+ cells ... 69
3.3.4 The role of Vitamin D may explain differing basal expression as well as unexpected programs of gene modulation between CD14+ cells and THP-1 cells ... 71

3.3.5 Overall Conclusions 73
4 CHAPTER FOUR: General Discussion and Conclusions 75
5 CHAPTER FIVE: Limitations and Future Studies 78
Bibliography 79
Appendix 86

Preface

The experimental work described in this thesis was conducted at the Africa Health Research Institute (formerly KwaZulu-Natal Research Institute for Tuberculosis and HIV- K-RITH), Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, from February 2015 to December 2016, under the supervision of Dr Emily Beth Wong and Prof Thumbi Ndung'u.

This work has not been submitted in any form for any degree or diploma to any other tertiary institution, where use has been made of the work of others, it is duly acknowledged in the text.

Abstract

Introduction: Tuberculosis is the leading cause of mortality in HIV-infected individuals worldwide. HIV-infected individuals have increased risk of developing active tuberculosis even at high CD4 T-cell counts and while undergoing effective antiretroviral therapy. The mechanisms that result in anti-TB immune dysfunction in HIV-infected people are not well understood. Previous studies have shown that a type II (IFN-γ) interferon-driven vitamin D-dependent antimicrobial pathway in monocytes and macrophages is important for the control of *Mycobacterium tuberculosis* (*M. tb)* growth *in vitro*. Notably, acute and chronic HIV infection are associated with high levels of type I interferons, specifically interferon alpha 2a (IFNα2a). Type I interferon has been shown to be upregulated during *M. tb* infection and has been found to be detrimental to *M. tb* control. We, therefore, hypothesised that HIV-1 infection induces an interferon-driven dysregulation of the vitamin D-dependent antimicrobial pathway in monocytes, which may predispose to tuberculosis susceptibility. The goal of this project is to lay the methodological

groundwork that will allow a comprehensive assessment of this hypothesis in a monocytic cell line and in primary human peripheral blood samples.

Methods: In this study, a one-plate quantitative polymerase chain reaction (qPCR) protocol for the reliable detection of Cytochrome P450 family 27 subfamily B member 1 (CYP27b1), cytochrome P450 family 24 subfamily A member 1 (CYP24a1), Cathelicidin (CAMP), Beta-defensin (DEFB4), and Vitamin D receptor (VDR) by the LightCycler 480 (Roche) was optimised for the human monocyte monocytic cell line (THP-1). The assays were also used to evaluate the expression of these transcripts in primary human peripheral CD14+ monocytes isolated using immunomagnetic separation from healthy donors (n=3). Cells were stimulated with recombinant human interferon gamma (rhIFNγ) (2,000 IU/mL) and recombinant human interferon alpha (rhIFN2a) (2,500 IU/ mL) for 24 hours. The impact of Type I and Type II interferons on gene expression were calculated using the Pfaffl equation to compare expression of genes of interest (normalised to GAPDH) between stimulated and unstimulated conditions.

Results: Quantitative PCR protocols were developed to reproducibly measure CAMP, VDR and CYP27b1 in THP-1 cells and primary human monocytes. Basal levels of these three transcripts were expressed at similar levels in THP-1 cells and primary monocytes relative to GAPDH, however, CYP27b2 was notably less expressed in CD14+ cells relative to THP-1 cells. DEFB4 and CYP24a1 could not be detected in THP-1 cells1s but were reproducibly detectable in primary human monocytes.

Comparative analysis of THP-1s and healthy donor CD14+ monocytes after stimulation with type I and II interferon showed varying modulation of the genes of interest. In THP-1s IFNα2a had the expected inhibitory effect on the three detectable transcripts of interest (CAMP, CYP27b1 and VDR). Stimulation with IFNγ resulted in the unexpected down-regulation of CAMP and CYP27b1. In healthy donor CD14+ monocytes stimulated with IFNγ, CAMP, CYP24a1, CYP27b1 and DEFB4 were all significantly upregulated. IFNα2a resulted in a trend towards up-regulation of CAMP, CYP24a1, CYP27b1 and DEFB4. VDR expression was not modulated by either Type I or II interferon stimulation in healthy donor monocytes.

Conclusions: Stimulation with exogenous recombinant type I/II interferon showed unexpected programs of gene modulation. Interferon gamma stimulations generally upregulated key genes in the vitamin D-dependent antimicrobial pathway, supporting the hypothesis that type II interferon signalling contributes to the protective upregulation of antimicrobial peptides. Unexpectedly, interferon alpha elicited a non-significant upregulation of all vitamin D-antimicrobial peptides and associated regulatory transcripts in healthy donor peripheral monocytes, a finding that contrasts to the reported literature and requires further exploration.

Our study revealed significant differences in the expression and modulation of key genes in the vitamin D-dependent antimicrobial pathway between immortalised THP-1 monocytic cells and primary human monocytes. It is possible that the undetectable levels of CYP24a1 and DEFB4 in THP-1 cells are due to lack of bioavailable vitamin D. This hypothesis is supported by the specific, detectable expression of both transcripts in CD14+ monocytes from healthy donors, which were presumably vitamin D replete. Modulation of vitamin D levels was outside the scope of this project and further study is needed to explore this issue.

Acknowledgements

I would like to take this opportunity to thank several people who have supported and guided me through this project.

Firstly, I would like to sincerely thank Professor Thumbi Ndung'u and Dr Emily Wong for all their patience, help and mentorship. Their example and guidance have been invaluable. To everyone in the Ndung'u Lab at the Africa Health Research Institute (formerly K-RITH), I would like to say a big thank you for taking the time to help and teach, as well as for many a laugh. To my family and friends, thank you for the constant encouragement, belief and support. I would like to thank Dr Laura Lozza and Professor Stefan Kaufmann

from the Max Planck Institute for Infection Biology for their advice and gracious hosting of me at their institute.

Last, but certainly not least, I would like to thank the University of KwaZulu-Natal, Africa Health Research Institute and my funders the National Research Foundation, Wellcome Trust, Connect Africa Scholarship Programme and the Howard Hughes Medical Institute for their funding as well as the monumental opportunity that has been offered to me.

Declaration

I, Mr Nathan Scott Kieswetter (9101175091088) declare that:

1. The research reported in this dissertation, except where otherwise indicated, and is my original research. All experimental procedures, analysis, and writing were carried out solely by myself.
2. This thesis has not been submitted for any degree or examination at any other university other than the University of KwaZulu-Natal.
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List of Figures

Figure 1: Worldwide Estimates of People Living with HIV, 2013 (WHO, 2014). Sub-Saharan Africa has the highest burden of HIV infection worldwide (±25 mil infections), followed by South-East Asia and then North and South America. 2
Figure 2: Estimated Deaths due to HIV/AIDS by WHO Region, 2013 (WHO, 2014). Africa has the highest estimated number of deaths due to HIV/AIDS worldwide. This is closely followed by India and the Americas. 4
Figure 3: Phylogenetic Diversity of HIV and SIV (Calef, 2008). 5
Figure 4: Global TB Incidence Rates, 2015 (World Health Organization, 2016a). 7
Figure 5: Estimated TB deaths excluding those attributed to HIV co-infection, 2015 (World Health Organization, 2016a). 7
Figure 6: Schematic of Risk of TB and Change in CD4+ Cell Count from Onset of HIV Seroconversion (Havlir et al., 2008). After HIV seroconversion and before the use of ART, TB risk increases exponentially with decreasing CD4 T cell count. After effective ART, this risk is lessened but never returns to baseline. 10
Figure 7: Estimated HIV Prevalence in New/Relapse TB Cases in 2015 (World Health Organization, 2016a). In southern Africa, upwards of 50% of new cases of TB are in patients already living with HIV. 11
Figure 8: Cytokine Storm-induced by Acute HIV-1 infection (Stacey et al., 2009). During acute infection, a cytokine storm is induced. Many cytokines persist at elevated levels through to chronic phase of infection. 12
Figure 9: Generalised cell model showing the all available TLRs and associated biochemical cascades during upon detection of pathogen/pathogen related antigen. 16
Figure 10: Incidence of TB acquisition in adults receiving the MVA84A vaccine relative to those who received placebo. There was no significance difference observed between the test group and the placebo group (Ndiaye et al., 2015). 18
Figure 11: Incidence of TB Acquisition in Infants receiving the MVA84A vaccine relative to those who received placebo. There was no significance difference observed between the test group and the placebo group (Tameris et al., 2013). 18
Figure 12: Crystal Structure of Cathelicidin (Vandamme et al., 2012). 21
Figure 13: Crystal Structure of Beta-Defensin 2 (Protein Data Bank, 2001) 22

Figure 14: Vitamin-D antimicrobial pathway and hypothesised regulation of transcription of antimicrobial peptides and key regulatory transcripts by type I and II IFNs (Modlin and Bloom, 2013). 23
Figure 15: Illustrated Diagram of Hypothesis. Type II IFN (IFNγ) is upregulated during mycobacterial infection and facilitates the metabolism of vitamin D and upregulates the downstream expression of protective antimicrobial peptides. This protection is abrogated via the upregulation of Type I IFN (IFNα2a) during HIV infection. 27
Figure 16: The Generalised process of traditional Polymerase Chain Reaction. 29
Figure 17: Generalised Schematic of Real-Time Polymerase Chain Reaction using Intercalating DNA Dye (SYBR Green) to Detect the Amplification of cDNA. 30
Figure 18: Checklist of Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al., 2009) 33
Figure 19: Bioinformatic Validation of Primers for Vitamin D Receptor using NCBI's Primer-BLAST. The online analysis allows for the detection of potential off-target amplicons, primer binding regions, specificity and GC content of both sense and antisense primer. 43
Figure 20: Primer 3 Plus Analysis for Secondary Structures, GC Content and 3' Stability for Vitamin D Receptor (Bottom Right) and an Example of an Inappropriate Primer Pair (Top Right) 43
Figure 21: Gel Validation of Amplified Genes of Interest Product Size via Gel Electrophoresis. The size of each band correlated well with the predicted product length. 44
Figure 22: Elimination of Primer-Dimer in for the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) qPCR via serial dilution of Primers. 45
Figure 23: Amplification Curve of Cathelicidin (CAMP) 46
Figure 24: Melt Curve Analyses of CAMP showing A) the Melting Curves and B) the Associated Melting Peaks. Non-specific amplification as a result of genomic DNA contamination can be observed at a melting temperature of approximately 86°C. 47
Figure 25: Amplification Curves of CAMP Post Additional DNase Treatment.. 47
Figure 26: Melt Curve Analyses of CAMP showing A) the Melting Curve and B) the Associated Melting Peaks Post Additional DNase Treatment showing a single specific product being amplified................... 48
Figure 27: Output from Agilent Bioanalyser 2100 Showing High-Quality RNA (RIN=8) Extracted using the RNeasy Plus Mini Kit in Conjunction with an Additional DNase Treatment. 49
Figure 28: Detection of DEFB4 in THP-1 Cells vs. a Reference cDNA Library. A) Amplification curves of DEFB4 in THP-1 cells. B) Melting Curves of DEFB4 in THP-1 cells. C) Melting peaks of DEFB4 in THP-1 cells. D) Amplification curves of DEFB4 in a reference cDNA library. E) Melting Curves of DEFB4 in a reference cDNA library. F) Melting peaks of DEFB4 in a reference cDNA library. 51
Figure 29: Reference Gene Validation Experiment. HPRT, GAPDH and 18S all found to be suitable reference gene candidates for downstream relative qPCR gene expression assays. 53
Figure 30: Basal Expression of Transcripts of Interest Relative to GAPDH is Variable in THP-1 Cells and CD14+ Healthy Patient Cells. 62
Figure 31: Modulation of Gene Expression via Type I (2500 IU/mL) /II (2000 IU/mL) Interferon Stimulation of THP-1 Cells over 24 hours (n=3). 63
Figure 32: Modulation of Gene Expression via Type I (2500 IU/mL) /II (2000 IU/mL) Interferon Stimulation of Healthy Patient CD14+ Monocytes (n=3). 65
Figure 33: Summarised Effect of Type I/II Interferon on the Expression of Key Antimicrobial Peptides and Regulatory Transcripts of the Vitamin D-Dependent Antimicrobial Pathway in THP-1 cells and Healthy Patient CD14+ Monocytes. Black arrows/bars equate to modulation or no effect of Type II IFN (IFNγ) respectively. Purple arrows equate to modulation of Type I IFN (IFNα2a) respectively. *p<0.05............ 74

List of Tables

Table 1: Final Optimized Primers Sequences and Associated Information 40
Table 2: Summary of Optimized Standard Curves for All Genes of Interest 50

List of Appendixes

Appendix 1: Conditions for the design of an Optimal Primer Pair for Specific Amplification 86
Appendix 2: Primer-BLAST Alignments of Final Selected Primer-Pairs: A) CAMP, B) VDR, C) CYP24a1, D) CYP27b1, E) DEFB4 and F) GAPDH 87
Appendix 3: Additional Gene and Primer Information 88

Abbreviations

CRF - Circulation Recombinant Forms

ATCC - American Type Culture Collection

AIDS - Auto Immunodeficiency Syndrome

BACT - Beta Actin

BLAST - Basic Local Alignment Search Tool

CAMP - Cathelicidin

cDNA - Complementary DNA

CYP24a1 - Cytochrome P450 family 24 subfamily A member 1

CYP27b1 - Cytochrome P450 family 27 subfamily B member 1

DEFB4 - Beta Defensin 2

DMSO – Dimethyl sulfoxide

DNA - Deoxyribonucleic Acid

IFNγ – Interferon gamma

IFNα2a – Interferon alpha 2a

gDNA – Genomic DNA

dNTP - Deoxy-nucleotide-tri Phosphate

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV - Human Immunodeficiency Virus

LAM - Lipoarabinomannan

LPS - Lipopolysaccharide

M.tb - *Mycobacterium tuberculosis*

MDR-TB - Multi-Drug Resistant Tuberculosis

mRNA - Messenger Ribonucleic Acid

ssRNA – Single stranded RNA

NEAA – Non-Essential Amino Acids

PAM - Phorbol 12-myristate 12 acetate

PCR – Polymerase Chain Reaction

PPD - Purified Protein Derivative

RPMI - Roswell Park Memorial Institute

RT-Reverse transcriptase/transcription

qPCR –Quantitative Polymerase Chain Reaction

Taq - Thermus aquaticus

TB - Tuberculosis

TDR-TB – Totally Drug Resistant Tuberculosis

TLR – Troll-Like Receptor

UBC – Ubiquitin C

VDR – Vitamin D Receptor

XDR TB – Extremely Drug Resistant Tuberculosis

PAMP – Pathogen-Associated Molecular Pattern

PRR – Pattern Recognition Receptor

CRAMP – Cathelin-related Antimicrobial Peptide

MSM – Men who have sex with men

LGBT – Lesbian, Gay, Bisexual and Transsexual

1. CHAPTER ONE: Introduction and Literature Review

1.1 Human Immunodeficiency Virus (HIV)

Human immunodeficiency virus, or HIV, is a lentivirus of the *Retroviridae* family. HIV specifically infects human immune cells expressing the CD4 protein on the cell surface (Dalgleish et al., 1984). This targeted infection leads to a progressive worsening of the immune system, allowing for the subsequent acquisition of opportunistic infections. In the absence of antiretroviral therapy, infection with the virus almost always results in the development of the acquired immunodeficiency syndrome (AIDS) (Sharp and Hahn, 2010). HIV is primarily transmitted via the exchange of infected blood or other bodily fluids. The most common routes of transmission are through sexual intercourse, contaminated needles during drug use and mother to child transmission during gestation and birth. Stigma and societal vulnerability have driven high rates of infection in certain key populations such as homosexuals, intravenous drug users, transgender people and adolescents. These key populations thus remain a significant driver of infection in high resource settings. However, in the sub-Saharan Africa setting, the HIV epidemic is a generalised epidemic primarily transmitted through heterosexual intercourse.

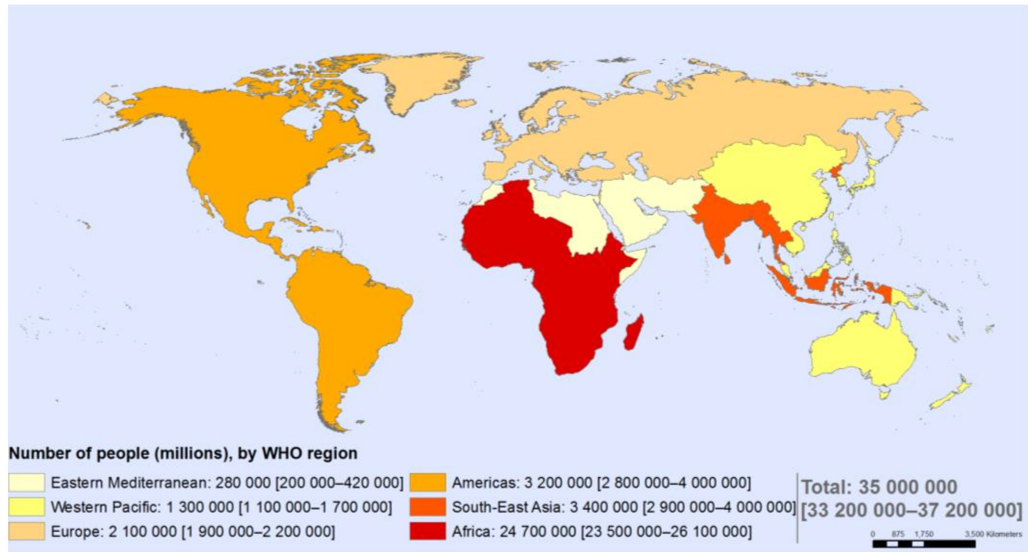


Figure 1: Worldwide Estimates of People Living with HIV, 2013 (WHO, 2014). Sub-Saharan Africa has the highest burden of HIV infection worldwide (±25 mil infections), followed by South-East Asia and then North and South America.

The discovery of HIV occurred approximately thirty years ago when doctors observed an unexplainable increase in the occurrence of Kaposi's sarcoma and other opportunistic infections in otherwise young, previously healthy, homosexual men in both New York City and Los Angeles (Barre-Sinoussi et al., 2013, Friedman-Kien et al., 1981). The predominant feature in the men was depression of adaptive and innate immune control during microbial challenge leading to increased incidence of opportunistic diseases and other microbial infections (Friedman-Kien et al., 1981). The occurrence of these opportunistic infections due to depressed immunity in predominantly gay men initially prompted the idea that this unexplained condition was only experienced by homosexual males. However, this was later proved not to be the case when similar symptoms were observed among intravenous drug users and blood transfusion recipients (Barre-Sinoussi et al., 2013). During this period, the condition was referred to as autoimmune deficiency syndrome, or AIDS, by the Centres for Disease Control (CDC) of the United States. In 1982, the first occurrence of mother to child transmission of AIDS was observed which led to the first known AIDS-related mortality of the child in question. The phenomenon of mother-to-child transmission

led scientists and doctors at the time to conclude that AIDS was caused by some unknown transmissible agent via blood and other possible body fluids rather than lifestyle choice (Notes, 1982).

The controversial idea that AIDS was caused by a retrovirus was first proposed to Robert Gallo, a prominent virologist, whose work led to the discovery of the human T-cell leukaemia virus (HTLV-1 and -2), the only known human retroviruses at the time (Montagnier, 2002). Gallo's group also pioneered the field in cytokine discovery by characterising and utilising interleukin 2 (IL-2) for the *in vitro* culture of human T-cells (Montagnier, 2002, Morgan et al., 1976). This provided the platform and opportunity to characterise hypothetical human T-cell tropic retroviruses *ex vivo*. This was later achieved by Luc Montagnier's research group at the Pasteur Institute in France (Montagnier, 2002). The group was fortuitously placed to tackle this particular question due to their interest in the search for lymphotropic retroviruses found in T cells (Barre-Sinoussi et al., 2013). In 1983, the first traces of reverse transcriptase were identified from a lymph node biopsy sourced from a homosexual male in the USA which was processed into a single cell suspension and cultured with T-cells using IL-2 (Montagnier, 2002). In the time between 1983 and 1984 Gallo's group, as well as Montagnier's group, published data firmly supporting the hypothesis that HIV was the cause of AIDS. This discovery accelerated the field of HIV research and allowed for the sequencing of the HIV genome in 1985, the identification of gp41 and the discovery that HIV-infected T-cells by using CD4 as its receptor (Montagnier, 2002, Dalgleish et al., 1984). Two years later, diagnostics for the detection of HIV-1 were developed, allowing screening of the blood supply to prevent transfusion-related transmission of the infection (Montagnier, 2002).

Structurally, HIV is generally spherical containing two single strands of RNA (ssRNA) encapsulated in a conical capsid composed of viral protein (Briggs et al., 2003). The two single-stranded RNA strands are bound to nucleocapsid proteins, viral proteins and enzymes critical for replication and maturation of HIV such as reverse transcriptase, integrase and protease (Briggs et al., 2003). Surrounding the viral capsid is a matrix composed of viral proteins p17 over which an envelope, which originates from

a host cell, containing glycoproteins gp120 and gp41 which enclose the entire capsid to form a mature infectious virion (Briggs et al., 2003).

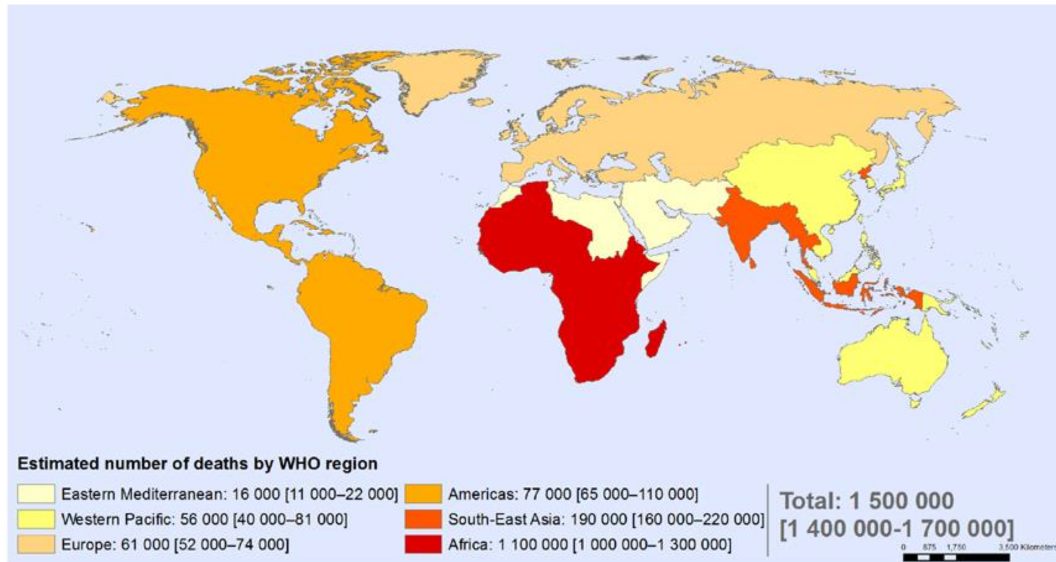


Figure 2: Estimated Deaths due to HIV/AIDS by WHO Region, 2013 (WHO, 2014). Africa has the highest estimated number of deaths due to HIV/AIDS worldwide. This is closely followed by India and the Americas.

Globally HIV affects more than 35 million people (Figure 1) with the bulk of the infections in developing countries, especially Africa and South Asia. In 2013, global deaths attributed to the HIV/AIDS were more than 1.5 million with the largest portion of AIDS-related mortality due to *Mycobacterium tuberculosis* the bacteria that causes tuberculosis (Figure 2).

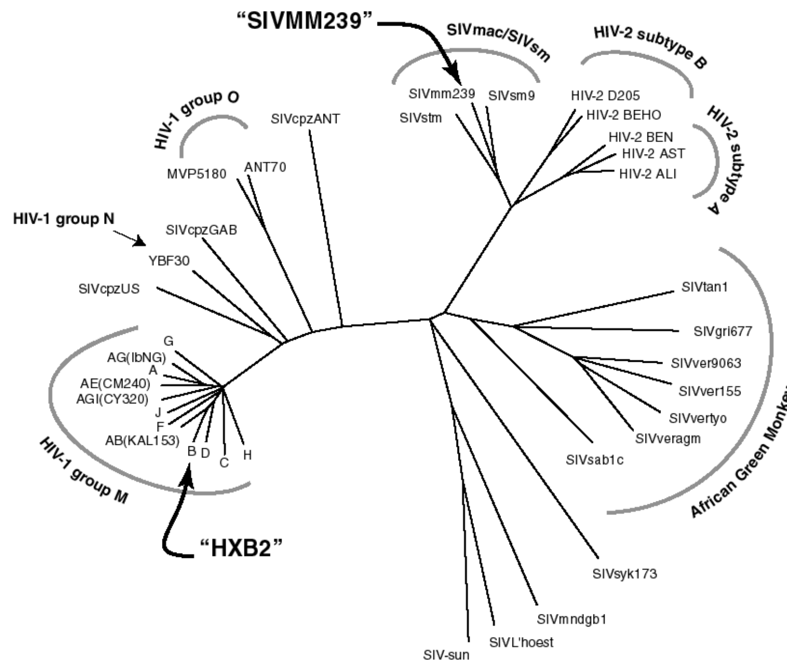


Figure 3: Phylogenetic Diversity of HIV and SIV (Calef, 2008).

HIV is characterised by both a high mutation rate, retroviral recombination and high replication rate which facilitates a high degree of genetic variability (Hemelaar, 2012, Smyth et al., 2012). As such, there exists a large diversity of HIV worldwide (Figure 3). There are two major types of HIV namely; HIV-1 and HIV-2. In general, HIV-1 has higher virulence, infectivity and is vastly more common worldwide representing approximately 95% of all infections (Sharp and Hahn, 2010). In contrast, HIV-2 is found mostly in West Africa and in areas with a historical link to West Africa (Hemelaar, 2012). HIV-1 can further be subdivided into groups M, N, O and P and in the case of group M, into subgroups (A-K) (Figure 3) (Hemelaar, 2012, Vallari et al., 2011). Each group of HIV-1 is hypothesised to represent independent transmissions of simian immunodeficiency virus (SIV) strains into human populations via non-human primate populations (Hemelaar, 2012, Vallari et al., 2011). Recombination of different functional subtypes is also common resulting in circulating recombinant forms, or CRFs, which further increase the diversity of HIV strains. Group M HIV-1 viruses are the most common, forming approximately 90% of cases and therefore primarily responsible for the acquired immunodeficiency syndrome pandemic. However, the HIV-2 virus can also result in AIDS (Hemelaar, 2012). The various subtypes are generally localised

geographically, however, increased global travel has allowed for the international spread of the various subtypes into previously non-endemic areas. Group N infection was initially observed exclusively in Cameroon in a small number of individuals who were tested (Hemelaar, 2012). Group O HIV-1 is also closely associated with the Cameroon and is rarely seen in regions of the world other than West Africa (Hemelaar, 2012). Group P HIV-1 virus was first identified in a woman living in France, who had previously lived in the Cameroon (Vallari et al., 2011). The viral sequence was found to be more similar to gorilla SIV rather than chimpanzee SIV (Hemelaar, 2012).

Fortunately, the development of combination antiretroviral treatment (cART), has significantly improved the prognosis of HIV-infected persons worldwide allowing them to lead long fulfilling lives (Smyth et al., 2012). However, the persistence of HIV in viral reservoirs even in the face of ART means that although the immune system (CD4 count) may be reconstituted to near normal levels, an infected individual will have to remain on ART for the remainder of their lives.

1.2 Tuberculosis (TB)

Mycobacterium tuberculosis, also known as Koch's bacillus, is a rod-shaped bacterium which was first discovered by Robert Koch in 1882 (Koch, 1882). It is a slow growing, aerobic bacterium which affects human hosts, and is not found in other species or abiotic environments (National Institute of Allergy and Infectious Disease, 2012). Tuberculosis (TB) is an ancient disease caused by a loss of host immune control of *Mycobacterium tuberculosis* (*M. tb*) and is the leading infectious killer worldwide (Muller et al., 2014, WHO, 2015, WHO, 2016a). *Mycobacterium tuberculosis* is an extremely infectious bacterium transmitted by inhalation of aerosolized respiratory secretions, with bacteria inhabiting tiny microscopic droplets of an *M. tb* infected persons' cough. As such, the risk of contracting *M. tb* infection is universal as it simply involves breathing.

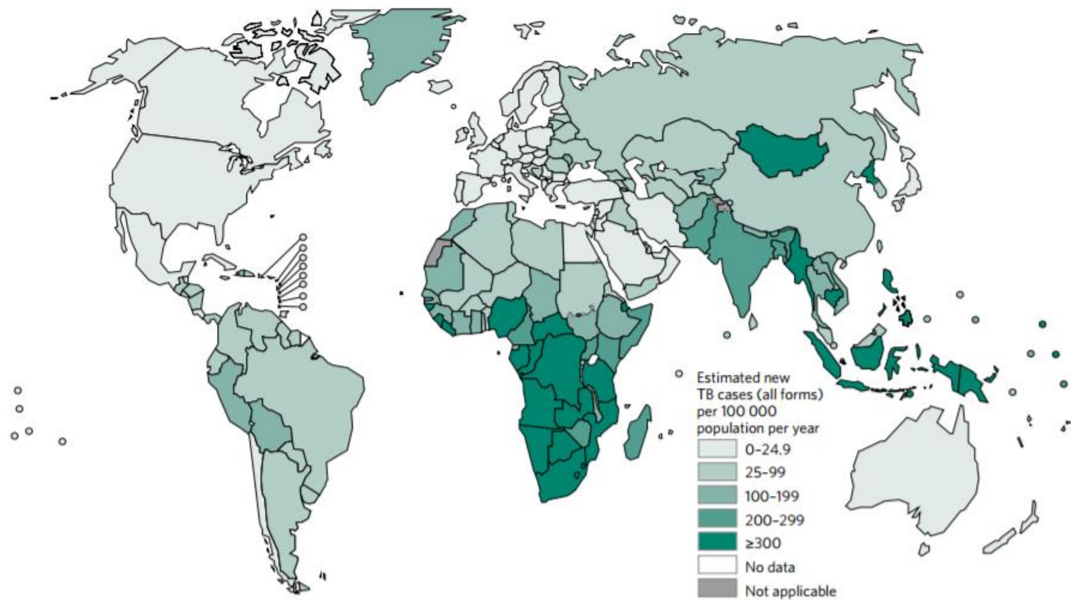


Figure 4: Global TB Incidence Rates, 2015 (World Health Organization, 2016a).

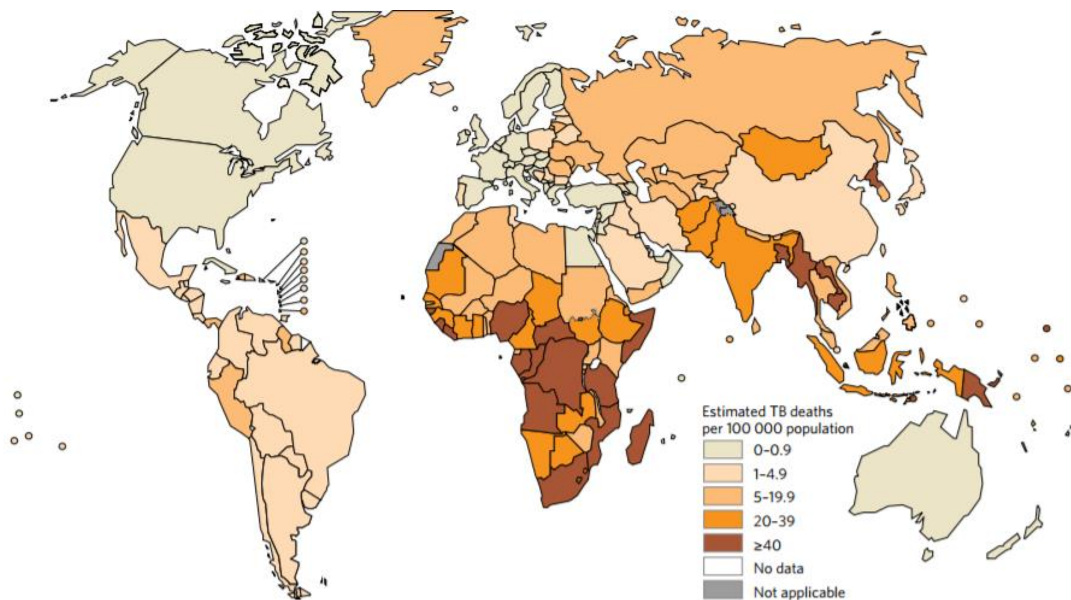


Figure 5: Estimated TB deaths excluding those attributed to HIV co-infection, 2015 (World Health Organization, 2016a).

Mycobacterium tuberculosis infection occurs worldwide; however, TB is most prevalent in developing countries in sub-Saharan Africa and South Asia (Figure 4). Figure 5 depicts estimated deaths

due to TB in 2015 (excluding cases in which the patients had HIV infection) demonstrating that the mortality burden is disproportionately borne by developing countries with particularly severe mortality rates in Central-Southern Africa and Asia. It is estimated that around 1.6-2.0 million people die from TB annually (WHO, 2016a). Globally, about 90% of tuberculosis cases occur in adults and 60% of TB mortality occurs in men. (World Health Organization, 2015, World Health Organization, 2016a).

Mycobacterium tuberculosis-infected persons who have not progressed to disease are termed ‘latently infected’ and have a positive tuberculin skin test (PPD Test) whilst having an absence of respiratory symptoms and negative sputum smears (Miller et al., 2000). In general, the bacterium can remain dormant and non-infectious in a healthy person for years and may become activated during immune suppression (Rappuoli and Aderem, 2011, National Institute of Allergy and Infectious Disease, 2012). It is estimated that more than two billion (approximately 1 in 3 people worldwide) people are infected with latent TB worldwide; of this 10 % will progress to active TB in their lifetimes (Rappuoli and Aderem, 2011, WHO, 2016a). The factors which lead to progression of latent to active disease are incompletely understood but include immune suppression (HIV infection, diabetes and tumour necrosis factor (TNF) neutralisation therapies) (Barry et al., 2009). In situations of repeated/prolonged exposure to environments in which a high degree of *M. tb* is present, such as clinics, prisons and countries with an extremely high prevalence of *M. tb* infection, it can be difficult to distinguish progression of latent infection from re-infection (Miller et al., 2000, National Institute of Allergy and Infectious Disease, 2012, WHO, 2015).

Once *M. tb* infection progresses to disease, it can infect different sites in the body causing pulmonary TB (in the lungs), TB meningitis (in the central nervous system) or disseminated TB (in any other region of the body e.g. bones, lymph nodes and the joints). The most common form of the disease in HIV-uninfected people is pulmonary TB in which *M. tb* infection results in lung cavities and presents with chest pain, coughing and blood in the sputum (Miller et al., 2000, National Institute of Allergy and Infectious Disease, 2012). Other symptoms include fever, night sweats and weight loss (Miller et al., 2000, National Institute of Allergy and Infectious Disease, 2012).

1.3 TB/HIV Co-infection

Recent genetic evidence suggests that TB has afflicted *homo sapiens* for as long as 15 000 years, whilst archaeological evidence suggests an even longer interaction (since the Neolithic period) supporting the hypothesis of historical coevolution (Muller et al., 2014). In the 1700-1900s, TB was a major cause of morbidity and mortality death for many areas of Europe where its rapid spread was facilitated via the high-density living conditions. The development of antimicrobial drugs coupled with improved public health practices resulted in a significant decline (approximately 45%) in the number of active tuberculosis cases in the 20th century.

The emergence of HIV/AIDS in the early 1980s led to a significant resurgence of active tuberculosis due to immune suppression. Globally, approximately 84% of new TB cases occurs in HIV negative individuals, however, in certain countries, HIV infection has been found to be major driver of new infection within these sensitive populations (WHO, 2016a). With relevance to this study, upwards of 50% of new cases of TB are reported among HIV-infected individuals irrespective of antiretroviral treatment status in Sub-Saharan Africa (Figure 7) (Rappuoli and Aderem, 2011, WHO, 2015, WHO, 2016a). Per the World Health Organization, TB is the greatest cause of death for HIV-positive populations worldwide and the deadliest infectious killer globally (Rappuoli and Aderem, 2011, WHO, 2015). In 2013 alone, it is estimated that approximately 9 million people worldwide developed TB of which 1.5 million people died of the disease (WHO, 2014). Of those, 360 000 people were HIV positive (WHO, 2014). The emergence of multidrug resistant (MDR) and extensively drug-resistant (XDR) phenotypes of TB in HIV-infected patients have further exacerbated the problem leading to an increase in morbidity and mortality worldwide in spite of modern drug treatment (Groschel et al., 2014, Brust et al., 2011). In 2015, 11% of all TB-related deaths in Africa and South East Asia were observed in HIV-positive individuals (WHO, 2016a).

Figure 6 illustrates the relationship between HIV infection, CD4+ cell count and TB risk over time following the onset of HIV infection. An inversely proportional relationship between CD4+ cell count and TB risk rate is observed over time, with TB becoming a progressively serious threat even when CD4+ cell

counts are still relatively preserved (>500 cells/μl) (Havlir et al., 2008). The exact mechanisms of how HIV exacerbates TB sensitivity remains a key gap in the knowledge of the field.

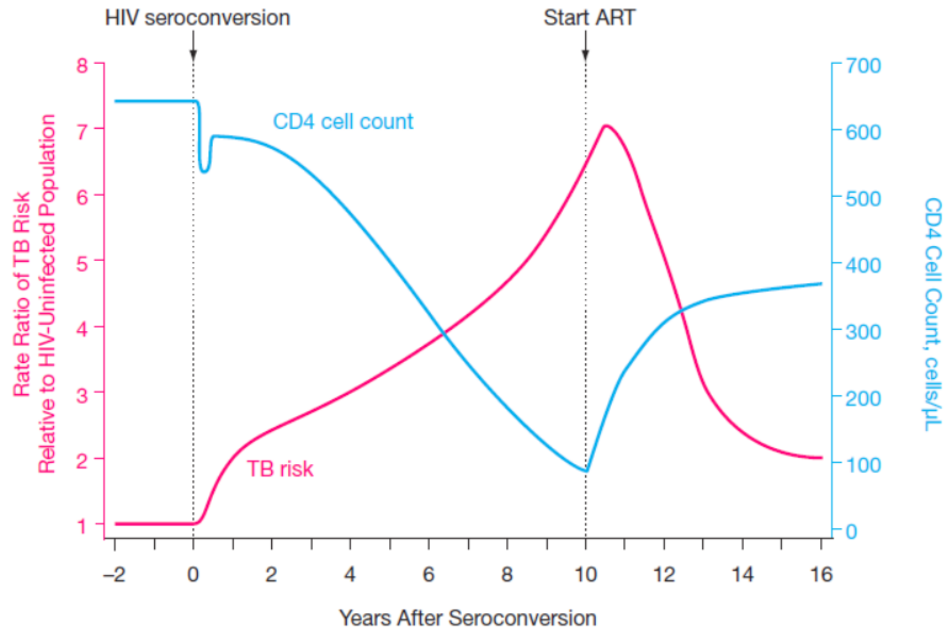


Figure 6: Schematic of Risk of TB and Change in CD4+ Cell Count from Onset of HIV Seroconversion (Havlir et al., 2008). After HIV seroconversion and before the use of ART, TB risk increases exponentially with decreasing CD4 T cell count. After effective ART, this risk is lessened but never returns to baseline.

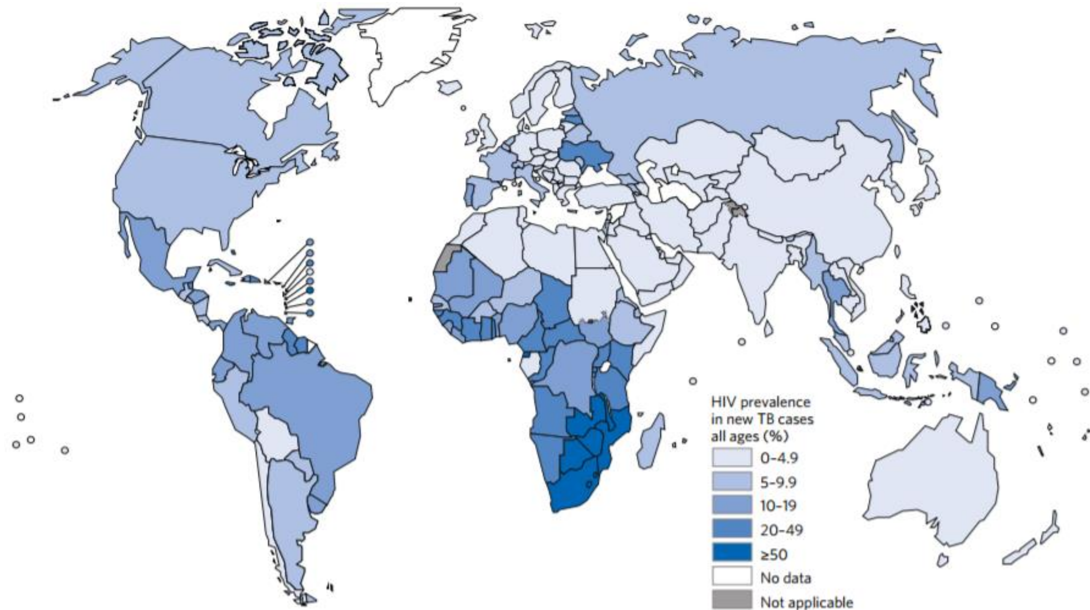


Figure 7: Estimated HIV Prevalence in New/Relapse TB Cases in 2015 (World Health Organization, 2016a). In southern Africa, upwards of 50% of new cases of TB are in patients already living with HIV.

TB remains the leading killer of HIV-infected individuals worldwide and much work remains to be done at both the political as well as basic research level to understand and stop the interactions of these two deadly infectious diseases.

1.4 Cytokines are associated with HIV infection.

During acute infection with HIV, the virus induces a global upregulation of immune regulatory cytokines in a phenomenon termed a "cytokine storm." (Figure 8) Although levels wane following acute infection, cytokines persist at elevated levels through chronic infection and never return to pre-infection baseline levels (Stacey et al., 2009). Cytokines are important cell signalling proteins which drive the ontogeny of immune cell populations as well as facilitate proper host immune responses, both humoral and cell-based. Cytokines include interferons (IFNs), interleukins (ILs), tumour necrosis factors (TNF) and chemokines.

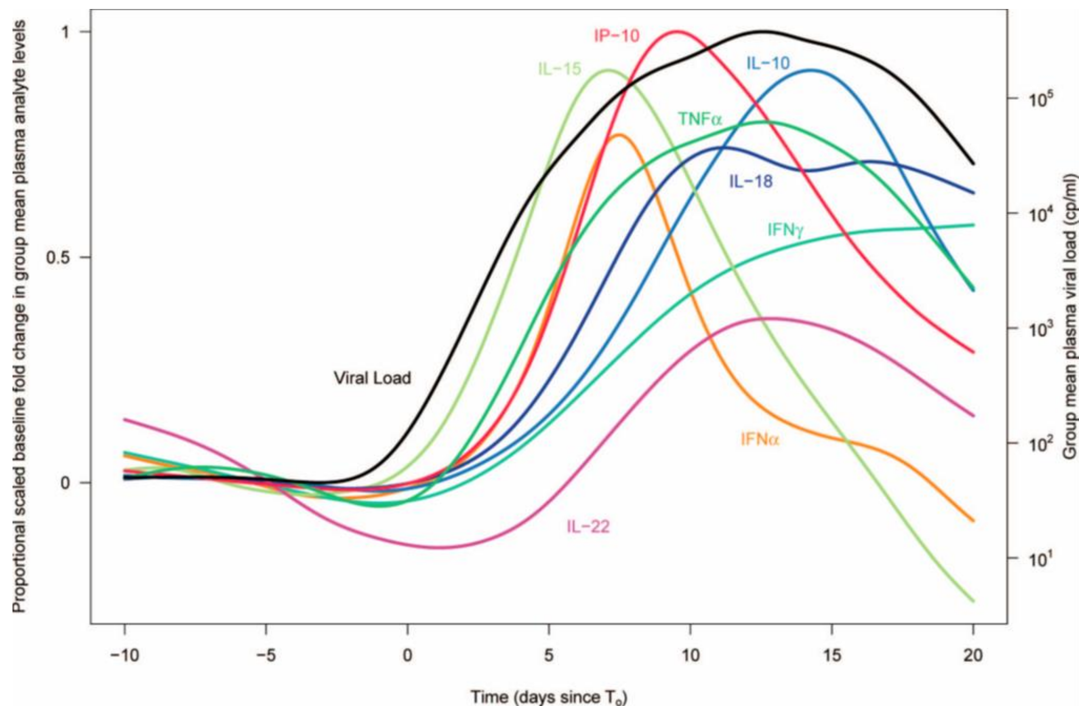


Figure 8: Cytokine Storm-induced by Acute HIV-1 infection (Stacey et al., 2009). During acute infection, a cytokine storm is induced. Many cytokines persist at elevated levels through to chronic phase of infection.

Of importance in mounting an effective immune response to invading pathogens is the induction of interferons during viral and bacterial challenge.

1.4.1 Interferon gamma (IFN-γ)

Interferons, as the name implies, are a diverse group of proteins which were initially discovered and named due to their ability to "interfere" with microbial infection, enabling clearance of pathogens by the host immune system (Schroder et al., 2004). Owing to varying homology and receptor binding sites, interferons are grouped into three groups; namely Type I, Type II and the recently discovered Type III (Schroder et al., 2004, Hermant and Michiels, 2014). Type III IFN has been implicated to be important in the antiviral response at barrier mucosal sites whilst avoiding the deleterious pathogenic risk associated with type I IFN antiviral activity (Wack et al., 2015). The effect or action of Type III interferons are beyond the scope of this thesis, as such, thus further discussion of interferons will be limited to the better understood Type 1 and Type 2 interferons. Historically, the two groups (IFN I/II) were thought to have distinct roles

in the innate immune system. Type II IFNs were associated with antibacterial/antimycobacterial activity and type I IFNs with antiviral activity. Although this is largely true, it is now known that most cases of pathogen clearance require a delicate, coordinated expression of both type I/II IFNs for an adequate immune response to be mounted.

There is only Type II interferon - interferon gamma (IFN-γ) which is expressed by CD4+ and CD8+ T cells. Natural killer cells and innate lymphocytes also express IFN-γ in response to bacterial infection (Schroder et al., 2004, Cooper et al., 2011). Knockout experiments in the murine model, for both IFN-γ and the receptor to which it binds, have been shown to cause no developmental defects (Huang et al., 1993). However, when challenged with viral and bacterial pathogens, there was a significant reduction in the resistance to infection (Schroder et al., 2004, Huang et al., 1993). This particularly true for mycobacteria infection in both mice models and studies of human families with inherited mutations in these sites (Cooper et al., 2011, Jouanguy et al., 1997, Jouanguy et al., 1996).

During acute HIV infection, the levels of IFNγ have been observed to significantly increase, and whilst they drop down as the infection progresses, the concentration in the periphery remains elevated in comparison to HIV-uninfected individuals.

1.4.2 Interferon alpha (IFNα2a)

Type I interferons were first described in a publication by Isaacs and Lindenmann nearly 52 years ago and have historically been primarily implicated in defence or protection during the viral invasion of the host (Trinchieri, 2010, Isaacs and Lindenmann, 1957, Hoofnagle et al., 1986). Intravenous IFNα2a treatment has been used successfully to treat a number of viral infections ranging from hepatitis B to human papillomavirus (HPV) (Nieminen et al., 1994, Hoofnagle et al., 1986). In fact, exposure of particular cells to this interferon induces a protective antiviral state which limits productive viral proliferation. Type I IFN is expressed by almost all cells in the body in response to free viral nucleic acid. There are 5 main types of IFN type I with multiple subtypes within each group. Of these, interferon alpha 2a, class II alpha helical cytokine, has been observed to be drastically increased in peripheral concentrations during acute HIV

infection and has been hypothesised to have significant implications for immune functioning during HIV/TB coinfection (Doyle et al., 2015, Gomez et al., 2015, Hardy et al., 2013, Ottenhoff et al., 2012). Like IFNγ, IFNα2a persists at an elevated level in people with chronic HIV infection which may modulate TB pathogenesis (Stacey et al., 2009).

In contrast to the generally protective role of type I interferons in viral infection, during bacterial infections, these interferons have a less clear role. Often, upregulation of type I interferon has a negative effect on many cells ability to control the infection of the invading pathogenic bacterium (Decker et al., 2005). However, this is not always true. In certain cases of bacterial infection, such as *Chlamydia trachomatis, Chlamydophila pneumoniae* and *Legionella pneumophilatype* type I interferon has been shown to inhibit replication *ex vivo* and thus abrogate pathogenesis both directly and indirectly (de la Maza et al., 1985, Buss et al., 2010, Plumlee et al., 2009). However, type I interferon is thought to fail to elicit protective responses against mycobacterial infection (Teles et al., 2013).

Exogenous application of type I interferon has been shown by several groups to encourage the growth of mycobacteria and exacerbate disease outcomes in human macrophages and monocytes (Bouchonnet et al., 2002). Redford *et .al* showed in mice that even temporary viral infection with influenza A correlated with an impairment of *M. tb* control in a type I IFN dependent manner, which may imply long-term impairment for patients suffering from chronic viral diseases such HIV-1 (Redford et al., 2014). In mice, inhibition of the IFNα2a receptor allowed for greatly reduced bacterial loads of *Mycobacterium tuberculosis* relative to wild-type mice and better disease outcome (Antonelli et al., 2010, Manca et al., 2001). Interestingly, in mouse models (severe combined immune deficiency (SCID)) infected with a hypervirulent clinical strain of *M. tb,* researchers reported significantly upregulated levels of type I interferon mRNA from dissected lung samples which was in turn correlated with increased mortality during early disease relative to wild-type mouse controls (Manca et al., 2001, Manca et al., 2005). These studies suggest that type I interferons may play a mechanistic role in the decreased anti-mycobacterial immunity that has been observed in the context of chronic HIV infection.

1.4.3 The Complex Interplay of Type I/II Interferon

Overall, it proper immune priming and clearance of invasive pathogens via the innate and adaptive immune system is facilitated through an interactive, coordinated expression of both types I and II interferons depending on the nature of the microbial challenge. The 'yin and yang' of type I/II IFNs, particularly the dysregulation of this balance during coinfection is hypothesised to affect the immune system's ability to clear a pathogen over the course of infection. The nature of interactions between these cytokines and its impact on infection outcome is still hotly debated (Decker et al., 2005). Further, the importance of interferon balance to the clearance of specific pathogens remains unclear. There is evidence that during certain infections, the two types of cytokines work synergistically to clear or control pathogens. In contrast, it has been demonstrated that in certain infections including mycobacterial, type 1 interferons may abrogate the protective effects of type II interferon leading to worsening of immune control and progression to clinical disease (Bouchonnet et al., 2002, Teles et al., 2013).

1.5 Toll-like Receptors (TLRs)

Toll-like receptors, or TLRs, are a varied group of membrane spanning proteins which can recognise a diverse range of chemical stimuli such as pathogen-associated molecular patterns (PAMPs) as well as chemical markers of tissue damage (Christmas, 2010, Kawai and Akira, 2010). Recognition of these molecules results in the establishment of the immune response, initially innate and later adaptive. These TLRs are found predominantly in cells most likely to be challenged by microbial pathogens, such as macrophages, monocytes, dendritic and other professional antigen presenting cells (APCs) which can mount an immediate innate immune response.

In mammals, thirteen TLRs have been discovered. In humans, nine TLRs are important, with TLR 11, 12 and 13 lost from the genome (Hidmark et al., 2012, Kawai and Akira, 2010). TLRs 1-2 and 4-6 are found on the outer surface of the cell and respond primarily to bacterial antigen stimulation. The remaining TLRs, 3 and 7-9, are found intracellularly on the surface of endosomes and primarily recognise pathogenic nucleic acids present during viral and bacterial infections (Figure 9) (Kawai and Akira, 2010). Upon the

binding of antigen to the pattern recognition receptor (PRR) of the TLR, two major signalling pathways become activated (Figure 9) (Kawai and Akira, 2010). The major pathway results in the activation of nuclear factor-κB and the mitogen-activated protein kinases p38 and JNK. The other pathway (primarily activated through TLR 3/4) allows for the activation of nuclear factor-κB as well as IRF3, which allows for additional anti-viral/bacterial genes to be activated resulting in the downstream expression of protective cytokines such as Type I interferons and other inflammatory cytokines (Kawai and Akira, 2010). These protective cytokines are essential for the modulation of successful innate and adaptive immune responses during microbial challenge (Kawai and Akira, 2010).

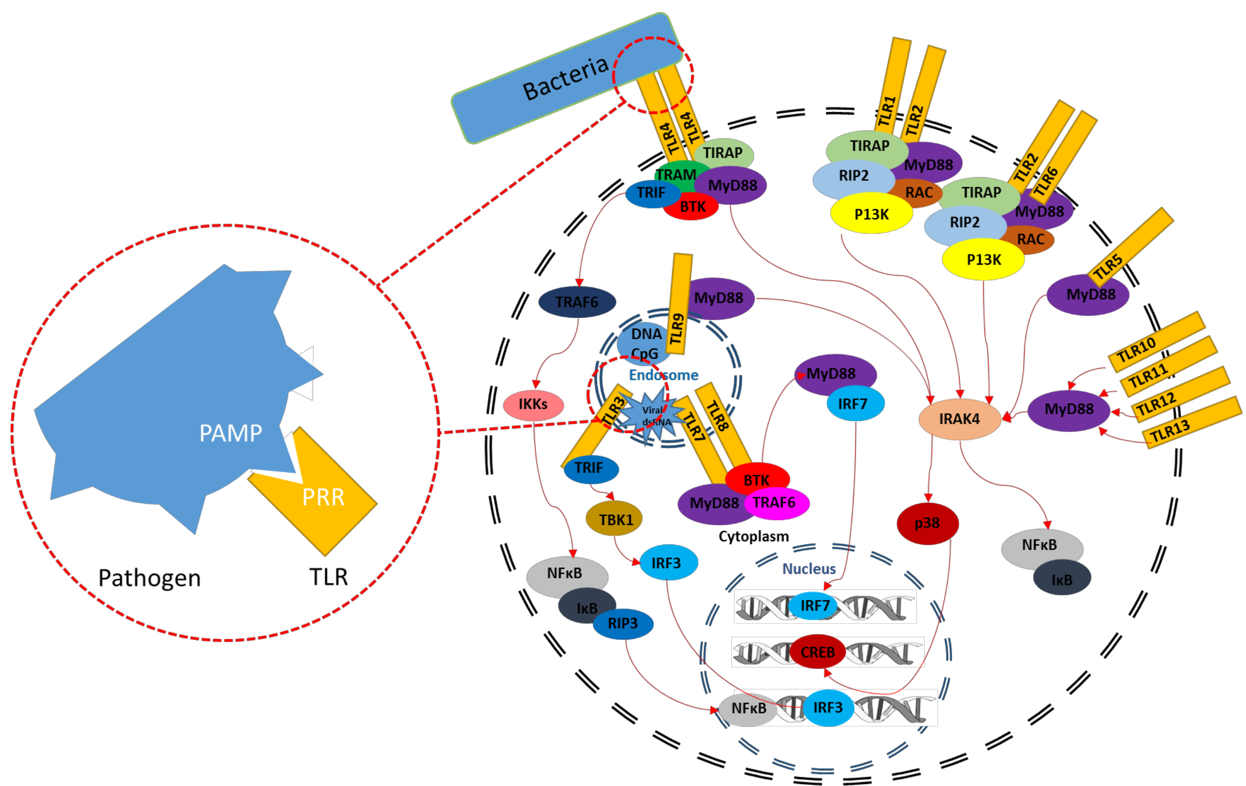


Figure 9: Generalised cell model showing the all available TLRs and associated biochemical cascades during upon detection of pathogen/pathogen related antigen.

1.6 Monocytes, Macrophages and the Innate Immune System

1.6.1 Challenges in TB vaccine design and the need to understand mechanisms of innate anti-TB immunity

A universally efficacious vaccine applicable to all age groups is urgently needed to prevent and control tuberculosis. Efforts to develop a novel vaccine have been hampered by the limited understanding of the mechanisms of protective immunity against *M.tb*. The Bacillus Calmette-Guerin (BCG) vaccine has been in use for many years with vaccinations carried out in early childhood and with varying success rate of 0 to 80% (Andersen and Doherty, 2005, Rappuoli and Aderem, 2011). Research has shown that the BCG vaccine does have the ability to protect against disseminated disease and death in young children and infants, however; protection against pulmonary disease was found to be highly variable (Andersen and Doherty, 2005, Rappuoli and Aderem, 2011). BCG however, is unable to protect adults against pulmonary or chronic infection (Rappuoli and Aderem, 2011).

The first new vaccine candidate since the discovery and implementation of BCG almost 50 years ago was an inoculant called MVA85A. (Ndiaye et al., 2015, Tameris et al., 2013). The candidate vaccine was synthesised from a strain of *Vaccina Ankara* virus which expressed antigen 85A from *M. tb.* Research on this vaccine candidate prior to clinical trials was promising, and MVA85A proceeded to clinical trials in both adults and infants (Ndiaye et al., 2015, Tameris et al., 2013). The vaccine was designed to be used as a viable boost following vaccination with BCG and was shown to have good immunogenicity in animal studies. Although the vaccine induced satisfactory cell-mediated host immune responses in both adults and infants, ultimately the vaccine was found to be non-protective as no significant difference was observed between the test treatment and the placebo (Figure 10 & 11) (Ndiaye et al., 2015, Tameris et al., 2013).

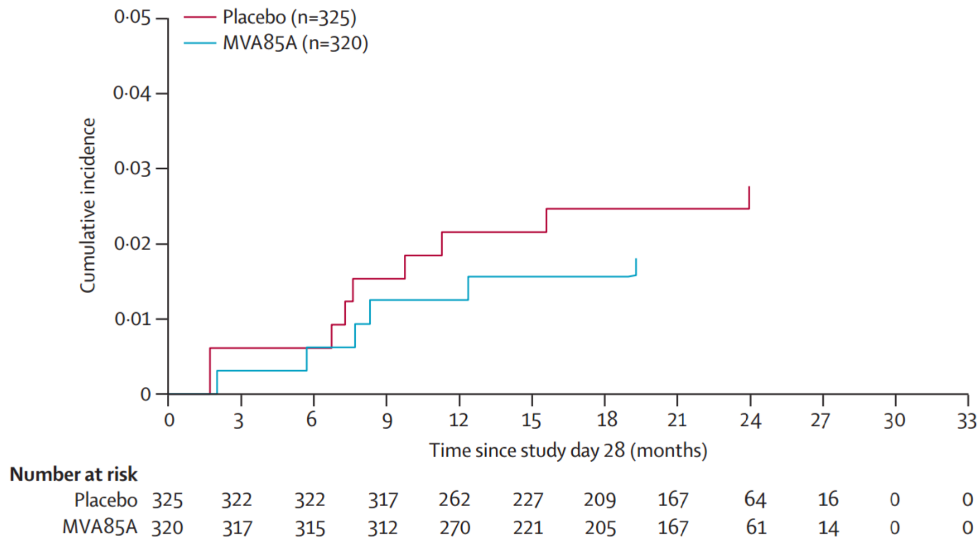


Figure 10: Incidence of TB acquisition in adults receiving the MVA84A vaccine relative to those who received placebo. There was no significance difference observed between the test group and the placebo group (Ndiaye et al., 2015).

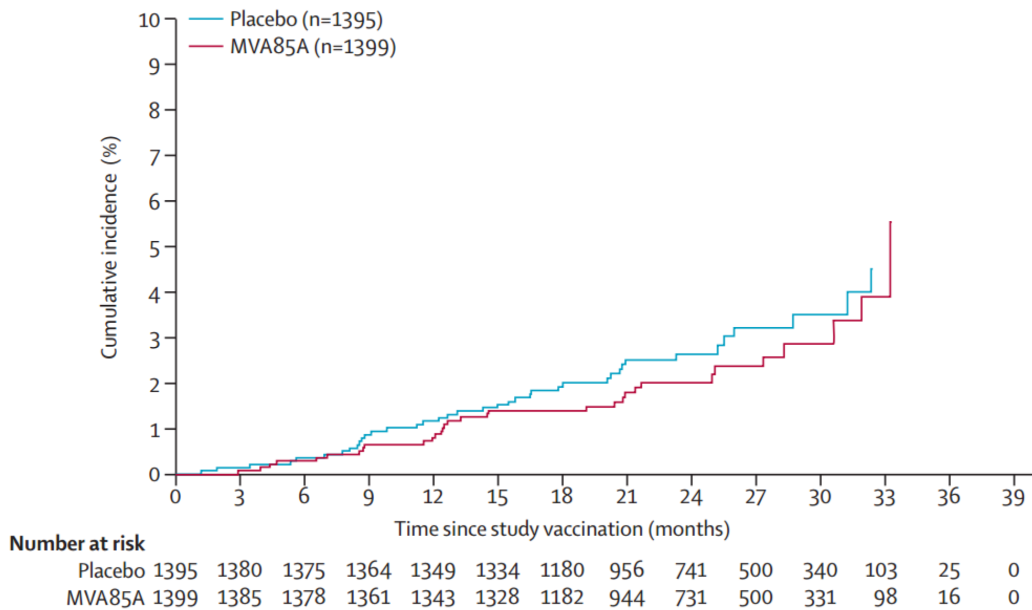


Figure 11: Incidence of TB Acquisition in Infants receiving the MVA84A vaccine relative to those who received placebo. There was no significance difference observed between the test group and the placebo group (Tameris et al., 2013).

The failure of MVA85a has highlighted the need for a better understanding of host defence mechanisms of natural anti-TB immunity. Animal studies suggest that innate immune cells such as macrophages and monocytes may play an important role in anti-tuberculous immunity (Modlin and Bloom, 2013). Furthermore, the observation that HIV-1 infected people, irrespective of disease stage or treatment status, are at significantly higher risk (26-30 times) for *M. tb.* infection and active disease suggest an irreversible loss of anti-TB immunity during coinfection with HIV (WHO, 2016b). The underlying mechanisms of this vulnerability remain largely undetermined. Thus, a better understanding of innate mechanisms of protective immunity against TB and a detailed understanding of the impact of HIV on anti-TB immunity may be a plausible pathway to the development of an effective vaccine or immunotherapy.

1.6.2 Vitamin-D Dependant Antimicrobial Peptides and their Role in Innate Immune Defence

Antimicrobial peptides or AMPs, (bactenecins, cathelicidins and defensins) are the ancient armaments of innate immune defence against microbial challenge in a number of species (Hazlett and Wu, 2011). There have been over eight hundred AMPs discovered from a range of living organisms, both prokaryotic and eukaryotic (Hazlett and Wu, 2011). In general, the peptides are named in accordance with their secondary structures; beta-stranded, alpha-helical, beta-hairpin and extended (Dhople et al., 2006). They are often called cationic AMPs due to the high density of positively charged amino acids present in their varying structures (Hazlett and Wu, 2011).

AMPSs are induced when the immune system recognises invading pathogens via toll-like receptors to control infection and prevent disease. They also have similar modes of protective activity despite their structural differences, with the positively charged portions of their structure interacting with the negatively charged regions on microbe cell walls/membranes leading to increased membrane fluidity and cell death (Hazlett and Wu, 2011). Thus, the expression and protective ability of these antimicrobial peptides depend largely on proper toll-like receptor activation post agonist recognition and the co-secretion of immunoregulatory cytokines by other cytokine-secreting leukocytes to mount an efficient immune response (Hazlett and Wu, 2011).

1.6.2.1 Cathelicidin (LL-37)

Cathelicidins are one of the primary arms of defence for prokaryotic and eukaryotic organisms. A distinguishing characteristic of cathelicidins is a much-conserved cathelin domain. Cathelicidin or as it known in its shortened forms; LL-37 or CAMP, is the only known human cathelicidin-derived antimicrobial peptide (Vandamme et al., 2012, Durr et al., 2006). The killing ability of cathelicidin, or LL-37, is largely due to its secondary structure (Vandamme et al., 2012, Durr et al., 2006). Cathelicidin interacts with the cell membrane of the pathogen either as a monomer or as complex oligomers. Lying parallel to the surface of the pathogen, cathelicidin binds to the negatively charged phospholipids on the surface of the pathogen. At a particular concentration of bound cathelicidin to the surface, positive curvature strain is induced. This is ameliorated by the movement of the cathelicidin peptides from the outer cell wall into the periplasmic region of the cell wall where cathelicidin then binds to the inner membrane of the pathogen. The movement of AMP from the outside of the cell to the inner membrane results in the formation of minute pores which disrupt the balanced homoeostasis of the membrane and cell wall biogenesis leading to cell death (Vandamme et al., 2012). Interestingly, cathelicidin has also been reported to have the effect of decreasing the ability of bacteria to infect cells by decreasing a cell's permeability (Byfield et al., 2011).

Cathelicidin has been shown in the literature to be effective against multiple microbial pathogens ranging from bacteria to fungi and is highly expressed at the site of inflammation during infection, thus this antimicrobial peptide is an important contributor in the clearance of invasive pathogens. Mouse cathelicidin knock-out studies reported a marked sensitivity to microbial infection, and increase in mortality and morbidity (Sonawane et al., 2011). To further substantiate the protective ability of LL-37, mice infected with drug-sensitive H37Rv and MDR strain of *M. tb* were dosed with human cathelicidin (LL-37 or CAMP), murine cathelicidin (CRAMP) and synthetic cathelicidin-like compounds. In all cases, the treatments reduced they mycobacterial load three to tenfold (Rivas-Santiago et al., 2013).

Cathelicidin has also been reported to be moderately protective against certain viral infections such as influenza, various adenoviruses and HIV-1. It has been shown that cathelicidin inhibits viral infection

through a direct interaction with the envelope region of an infective virion. Certain fragments of LL-37 are also able to directly block the ability of HIV-1 to reverse-transcribe its RNA (Wong et al., 2011).

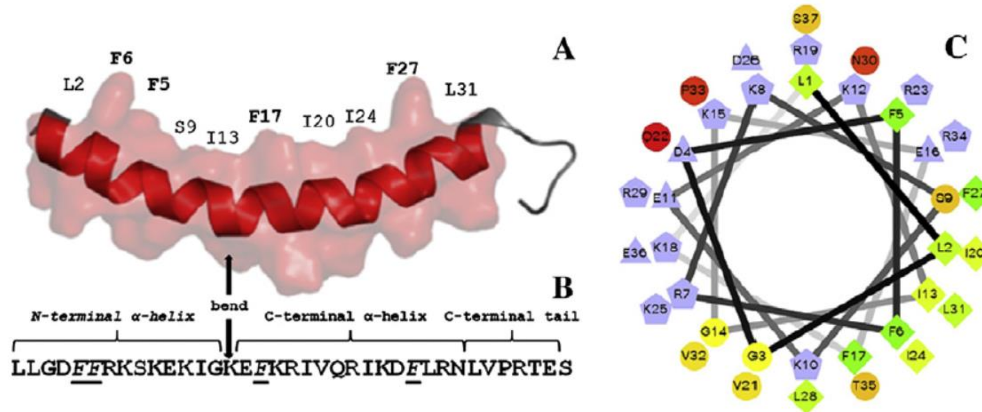


Figure 12: Crystal Structure of Cathelicidin (Vandamme et al., 2012).

1.6.2.2 Beta-Defensin 2

Defensins are cationic cysteine-rich peptides of relatively small size and are characterised by a beta-sheet structure bonded by three disulphide bonds formed of 6 cysteine residues (Figure 6) (Hazlett and Wu, 2011). Although structurally similar, defensins can be segregated into two main groups namely, alpha and beta defensins (Hazlett and Wu, 2011). Alpha-defensins are abundant primarily in the granules of neutrophils, whilst beta-defensins are slightly larger peptides which are produced in mucosal epithelial cell populations such as macrophages, monocytes and dendritic cells (Hazlett and Wu, 2011). Defensins are highly water soluble; however, they also have the ability to interact with the hydrophobic lipid structure of invading bacteria/mycobacteria (Hazlett and Wu, 2011).

During microbial challenge, innate immune cells very rapidly release defensins at the site of pathogen recognition. The rapid secretion of defensins into the cellular environment is hypothesised to limit pathogen replication/invasion as well as facilitate downstream adaptive immune responses (Hazlett and Wu, 2011). Twenty-eight different beta-defensin genes are thought to exist within the human genome; however very few have been adequately studied (Hazlett and Wu, 2011). In general, human beta-defensin

1 to 4 remain the most researched antimicrobial peptides of this type. Beta-defensin 2/3, in particular, are upregulated by bacterial challenge or their products such as lipopolysaccharide (LPS) and other cytokines such as TNF-α (Hazlett and Wu, 2011).

Ex vivo experiments involving the transfection of human monocyte-derived macrophages with mRNA encoding beta-defensin 2 enhanced the macrophages' ability to restrict the growth of *M. tb*, suggesting a role for DEFB4 in mycobacterial defence.(Kisich et al., 2001).

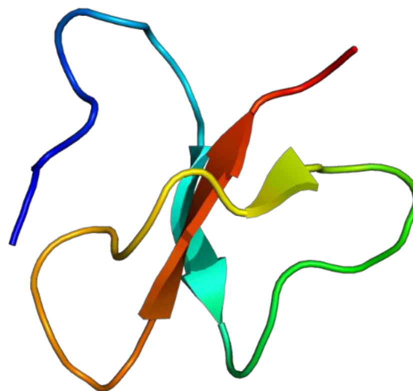


Figure 13: Crystal Structure of Beta-Defensin 2 (Protein Data Bank, 2001)

The expression of both cathelicidin and beta-defensin 2 are under the control of a Vitamin-D dependent antimicrobial pathway in a range of phagocytes (Modlin and Bloom, 2013, Liu et al., 2006, Liu et al., 2007).

1.6.3 Innate Protection: Vitamin D Dependent Antimicrobial Pathway

The innate immune response is the body's first response to invading pathogens. It can be thought of as having three main arms which provide initial and immediate protection; physical, cellular and humoral (Hazlett and Wu, 2011). Protective barriers such as the skin or other epithelial layers constitute the physical aspect of innate protection and the first major obstacle which needs to be overcome by an invading pathogen to achieve successful infection (Hazlett and Wu, 2011). Humoral protection is facilitated via the secretion of protective cytokines/chemokines which act directly against pathogens, restricting replication and often

facilitating cell death (Hazlett and Wu, 2011). Cellular innate control is the most important aspect of innate immunity allowing for the control and clearance of invading pathogens (Hazlett and Wu, 2011). Innate immune cells phagocytose microbes and eliminate them via enzymatic means or through the action of antimicrobial peptides (Hazlett and Wu, 2011). Phagocytes able to degrade pathogens can then present processed microbial peptide fragments on the surface of their cells for recognition by the adaptive immune system.

Of particular importance in defence against *M. tb* is the action of circulating monocytes and tissue-resident macrophages in the lung (alveolar macrophages) which generally are the first cells to interact with a pathogenic aerosol and have the ability to control infection in healthy individuals. It is hypothesised that in some individuals who are highly exposed to TB patients, the innate response at respiratory mucosa may be sufficient to prevent infection as indicated by long-term negative skin tests (TST) (Modlin and Bloom, 2013). It is possible that the vitamin D-dependent antimicrobial pathway may contribute to control in these high risk, yet healthy individuals (Figure 14).

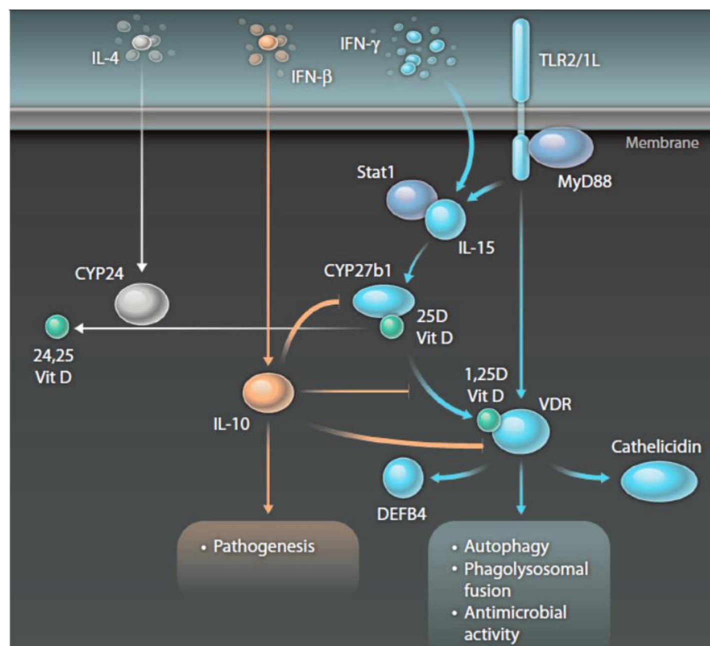


Figure 14: Vitamin-D antimicrobial pathway and hypothesised regulation of transcription of antimicrobial peptides and key regulatory transcripts by type I and II IFNs (Modlin and Bloom, 2013).

Macrophages and monocytes are phagocytic, large white blood cells which are important components of the innate immune response to *M. tb* (Liu et al., 2007, Maess et al., 2010). These phagocytes have the ability to engulf or phagocytose foreign particles and pathogens such as bacteria and mycobacteria (Maess et al., 2010). Once engulfed, the foreign body is sequestered in a membrane-bound vacuole called a phagosome (Aderem and Underhill, 1999). The phagosome fuses with a second membrane-bound organelle filled with degradative enzymes and other antimicrobial molecules, called a lysosome to form the phagolysosome (Aderem and Underhill, 1999). This joining results in the degradation of foreign particles, but is countered by of the ability of phagocytosed *M. tb* to inhibit phagolysosome fusion (Aderem and Underhill, 1999). Nonetheless, *in vivo* and *ex vivo* experiments have demonstrated the ability of macrophages and monocytes from healthy individuals to control *M. tb* infection (Demissie et al., 2004, Modlin and Bloom, 2013). This control of infection is thought to be at least partly mediated through the production of antimicrobial peptides for the destruction of internalised mycobacteria (Modlin and Bloom, 2013, Agerberth et al., 2000).

Mouse studies have shown that the main arm of antimicrobial clearance in murine macrophages is facilitated via nitric oxide synthase (Chan et al., 1995, MacMicking et al., 1997). Conversely, *ex vivo* human studies imply that the innate immune response during microbial challenge is mainly facilitated through the production of antimicrobial peptides rather than ROS-burst (Modlin and Bloom, 2013). This monocyte/macrophage antimycobacterial activity is partially mediated by Toll-like receptor (TLR2/1), vitamin D-dependent induction of potent antimicrobial peptides, cathelicidin and β-defensin 2 (DEFB4) (Modlin and Bloom, 2013, Campbell and Spector, 2012, Chun et al., 2015) (Figure 8). The Vitamin-D antimicrobial pathway (Summarized by Modlin and Bloom (2013) in Figure 14), involves the following key steps: (1) activation of TLR1/2 leads to the upregulation of CYP27b1 which converts 25-hydroxyvitamin D (25D) to the bioactive form 1,25-dihydroxyvitamin D (1,25D) (2) upregulation and

activation of the vitamin D receptor resulting in (3) the production of CAMP and DEFB4 to mount an effective immune response (Modlin and Bloom, 2013, Adams et al., 2009).

These antimicrobial peptides are in turn partially modulated by type II interferon, IFN-γ, which is mainly secreted by T-cells during bacterial infection (Modlin and Bloom, 2013). Thus, effective immune control of *M. tb*, requires a robust T-helper type 1 (T_h1) immune response, which has both a direct anti-TB effect but also influences the accompanying innate immune responses of macrophages and other antigen presenting cells (Montagnier, 2002, Teles et al., 2013). Indeed, current tuberculosis immunotherapy treatments and research are being aimed at the encouragement of T_h1 responses to facilitate the efficient management of infection (Groschel et al., 2014). Both arms of the immune response are thus thought to act synergistically for effective control of mycobacterial infection.

However, viral infection, especially HIV, is a known potent inducer of type I interferons such as IFN-α and IFN-β which may lead to downregulation of Type-I-interferon mediated T cell responses and downstream signalling pathways such as those that lead to potent induction of anti-microbial peptides in macrophages (Decker et al., 2005, Teles et al., 2013). Furthermore, HIV-1 infection is associated with the induction of a cytokine storm in acute HIV-1 infection, with the elevated secretion of some cytokines, notably type I interferon, persisting through the chronic infection phase (Roberts et al., 2010, Stacey et al., 2009). The impact of these cytokines or of HIV-1 proteins on anti-TB immunity has not been fully characterised and remains a key gap in knowledge in the field.

1.7 Aim of Study

Study of antimicrobial peptide expression in the face of type I/II interferon imbalance has the potential to lead to a more accurate understanding of the mechanisms underlying innate immune response dynamics in TB-HIV coinfection. Such knowledge may eventually facilitate better treatment for patients suffering from TB/HIV coinfection worldwide. The aims of this project were as follows;

Firstly, to optimise RT-qPCR assays to quantitate levels of transcription of key components of the antimicrobial peptide pathway: CYP27b1, CYP24, Cathelicidin (CAMP), Beta-defensin (DEFB4), and Vitamin D receptor (VDR).

Secondly, utilising primary CD14+ monocytes from healthy individuals, to assess the range of normal expression of these transcripts relative to the basal expression observed in the immortalised monocytic cell line, THP-1 cells.

Thirdly, to assess the impact of recombinant human type I/II interferon on the production of the transcripts of interest in THP-1 cells and CD14+ healthy patient monocytes.

1.8 Hypothesis

We hypothesised that elevated levels of Type-I IFN (such as induced in viral infections like HIV-1) result in decreased production of the Vitamin-D dependent antimicrobial peptides (Figure 15). Specifically, we hypothesise that exogenous application of Type-I IFN (at a physiologically relevant concentration to that seen in HIV infection) to innate phagocytes *ex vivo* will downregulate key antimicrobial peptides. If confirmed, this would provide a potential mechanism by which HIV-1 infection abrogates the innate immune system's control of latent *M. tb* infection.

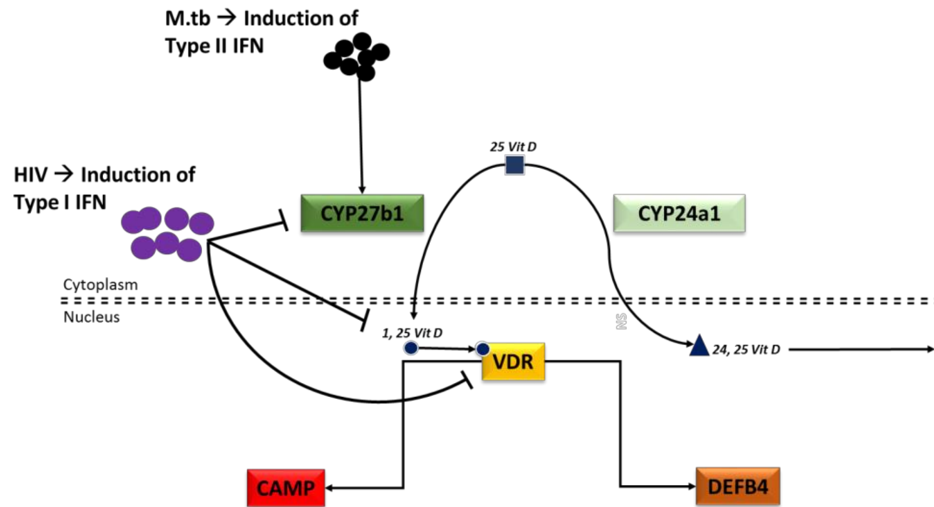


Figure 15: Illustrated Diagram of Hypothesis. Type II IFN (IFNγ) is upregulated during mycobacterial infection and facilitates the metabolism of vitamin D and upregulates the downstream expression of protective antimicrobial peptides. This protection is abrogated via the upregulation of Type I IFN (IFNα2a) during HIV infection.

1.9 Rationale

South Africa has one the highest prevalence of HIV infection in the world. People suffering from HIV are 30-40 times more likely to develop TB. Further, tuberculosis remains the greatest killer of HIV-infected people worldwide. By gaining a better understanding of host defensive mechanisms, such as the vitamin D-dependent pathway, novel host-focused therapeutic treatments/vaccines may be designed to help the patient better control *M. tb* infection. Adjunctive immunotherapies have the potential to ameliorate the symptoms of TB or improve the speed of eliminating infection in conjunction with standard TB treatment.

1.10 Specific Objectives

The specific objectives of this study are as follows;

Firstly, to optimise real-time PCR assays for CAMP, VDR, CYP24a1, CYP27b1, DEFB4 and GAPDH in the THP-1 cell line for accurate, repeatable quantitation of relative gene expression.

Secondly, to compare the basal expression of CAMP, VDR, CYP24a1, CYP27b1, DEFB4 in THP-1 cells relative to CD14+ cells from healthy donors.

Thirdly, to purify CD14+ cells (monocytes) from healthy donors to assess how gene expression of CAMP, VDR, CYP24a1, CYP27b1, DEFB4 is modulated by stimulation with recombinant human type I interferon (rhIFNα2a) and recombinant human type II interferon (rhIFNγ) in THP-1 cells and CD14+ human monocytes.

2. CHAPTER TWO: Optimisation of qPCR Assays for Reliable Detection of Transcripts of Interest in the Vitamin-D Dependant Antimicrobial Pathway in the THP-1 Cell Line

2.1 Brief Introduction

Accurate detection of Vitamin-D dependent antimicrobial transcripts (CAMP and DEFB4) and associated regulatory genes (VDR, CYP24a1 and CYP27b1) requires an optimised and validated gene expression assay. Real-time PCR has been widely regarded as the gold standard for the quantification of genes since its establishment in the early 1980s. PCR has the benefit of being able to amplify low quantities of DNA (theoretically down to one copy) whilst avoiding the quantification of easy-to-degrade proteins which may affect assay accuracy. It also facilitates the ability to quantify many varying factors or genes at once. The success of this technology is a testament to its routine use in many different industries ranging from forensics to diagnostics. Even with the advent of modern transcriptomic technologies such as RNA sequencing (RNA-seq), qPCR remains a key technology for the validation of high throughput hypothesis generating technologies.

2.1.1 Traditional PCR vs. Real-Time PCR

Quantitative real-time polymerase chain reaction (qPCR) is an improvement on the traditional PCR first pioneered by Kary Mullis in 1983 (Kubista et al., 2006, Kozera and Rapacz, 2013). PCR utilises a pair of artificially created DNA oligonucleotides (called primers) which flank a target single-stranded DNA sample sequence, the enzyme polymerase to amplify the region in-between the primers (Kubista et al.,

2006) and cycles of heat to dissociate double-stranded DNA and promote amplification. PCR generally has three phases; denaturation, annealing and elongation. The sample is heated to a particular temperature to facilitate the denaturation of double-stranded DNA to single-stranded DNA. The annealing step allows for the attachment of the primers to the single-stranded template. Finally, the elongation step entails the extension of the product via polymerase using the position of the primers to define the intended product (Kubista et al., 2006). This process is then repeated for a number of thermal cycles allowing for the exponential amplification of the targeted DNA sequence (Figure 16) (Kubista et al., 2006). The reaction eventually plateaus when the reagents in the reaction are used up (Kubista et al., 2006). A 100% efficient PCR reaction will result in the exponential doubling of product with every cycle.

As with conventional PCR, qPCR also involves the exponential replication of small samples of DNA utilising primers, dNTPs and *Taq polymerase*. However, via the use of intercalating dyes (such as SYBR green I) or hybridization probes (*Taq*Man®), cycles of amplification of targeted DNA can be measured in real time (following each thermal cycle) via the measurement of fluorescent signal as a measure of the exponential amplification of the target sequence (Figure 17) (Kubista et al., 2006, Heid et al., 1996). This allows for the quantification of gene expression levels under particular conditions. The amplified product can also be run on an agarose gel after the qPCR has been concluded, allowing for validation, sequencing, cloning and possible transformation.

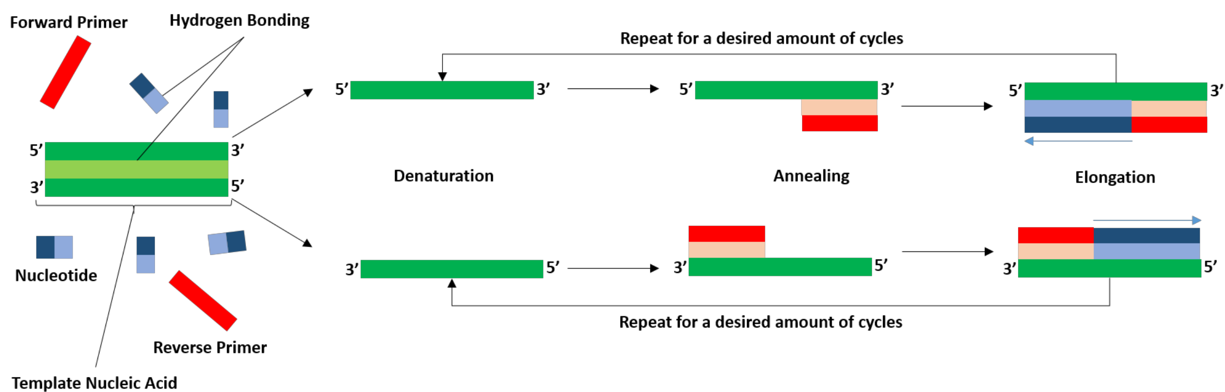


Figure 16: The Generalised process of traditional Polymerase Chain Reaction.

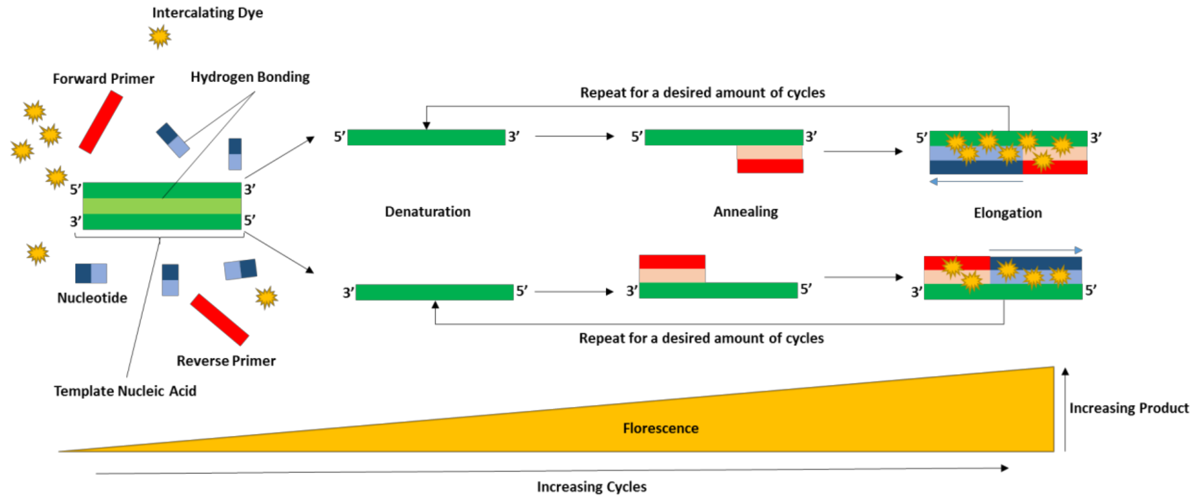


Figure 17: Generalised Schematic of Real-Time Polymerase Chain Reaction using Intercalating DNA Dye (SYBR Green) to Detect the Amplification of cDNA.

2.1.2 Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines

Since the advent of real-time quantitative PCR, many studies have used this technology as a platform for discovery. It has been extensively used in almost all aspects of biology from agriculture to clinical diagnostics. This is reflected in the numerous publications which have presented qPCR data, with most studies using varying amplification conditions, differing kits, primers, chemistries, and varying methods of nucleic acid extraction. As such, there has been a lack of consensus on how to optimally run and analyse qPCR experiments and data. This had an adverse effect on assay reproducibility and transparency. (Bustin et al., 2009). To remedy this, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments, or MIQE guidelines, were published by Stephen Bustin *et al.* in 2009. The paper's objective was to provide a general set of guidelines or minimum criteria to which all studies utilising qPCR should adhere to promote transparency and reproducibility (Figure 18). These guidelines have now become the standard for all studies utilising real-time PCR. Many biotech companies have incorporated the guidelines into the software of their products to make the standardisation of

experimental data to the guidelines easier. These guidelines were followed in the optimisation of the qPCR protocols for this project.

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Inhibition testing (C_q dilutions, spike, or other)	E	r^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[c]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

[a] All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.

[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.

[c] Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as rDNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.

[d] Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

Figure 18: Checklist of Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al., 2009)

2.1.3 Optimisation of Issues Encountered in Real-Time PCR

There are several experimental obstacles that need to be optimised for a successful qPCR protocol to be established;

2.1.3.1 Primer-Dimer

Primer-dimer refers to the preferential binding of the forward and reverse primers to one another rather than the intended target sequence. This occurs due to the high occurrence of complementary nucleotide bases between the primers leading to hybridization to form a double-stranded product. This unintended by-product of a poorly optimised PCR protocol will result in the competitive usage of reaction reagents, thus decreasing the ability of the reaction to efficiently amplify the target DNA sequence. If present, primer-dimers can decrease the efficiency of a given PCR reaction, thus skewing the results for accurate quantitative analysis of the transcript of interest. Primer-dimers generally range from about thirty to fifty nucleotide bases in length and are identified by a melting peak at a lower temperature to that of the target amplicon. In an optimised qPCR protocol, the elimination of primer-dimer is an essential step (Raymaekers et al., 2009).

2.1.3.2 Non-Specific Amplification

Non-specific implication refers to the off-target amplification of an unintentional product during a PCR. This occurs when the PCR primers are complementary to other unintended regions on the template DNA, thus resulting in one or more products being amplified in addition to the target of interest. Non-specific amplification can be a result of two main causes. First, poorly designed primers can result in unintended amplification or primer dimer. Online bioinformatics tools such as NCBI's Primer-BLAST have been developed to allow assessment of the binding characteristics of a theoretical primer before it is manufactured, saving time and money. Secondly, non-specific amplification in gene expression experiments can be caused by genomic DNA contamination of the copy DNA (cDNA) library. Such

contamination often occurs when manoeuvres to remove genomic DNA from extracted RNA (such as DNase treatment or DNA binding) are inefficient.

2.1.3.3 Reference Gene Validation

Relative quantification of gene expression profiles using real-time PCR requires the use of robust endogenous reference genes for the adequate normalisation of qPCR data (Kozera and Rapacz, 2013). A reference gene is a particular gene whose expression remains constant independent of stimulation or activation, genes fulfilling "housekeeping" functions are often the best candidates. An ideal reference gene allows for the normalisation of gene expression data to cell number (Kozera and Rapacz, 2013). In a fully optimised relative qPCR protocol, the reference gene should be stably expressed regardless of experimental treatment for a specific number of cells. Some reference genes, however, may be modulated by experimental stimulations or tissue and will influence the accuracy of the quantification of gene expression. As such, it is essential to validate several potential reference genes to assure that they are stably expressed in the cell type being used in the experiment.

2.1.3.4 Differential Expression between Cell Types

Expression of a transcript can be modulated by many different factors such as media type, cell type, the state of activation, culture conditions and others factors. Consideration must be given to the cell type used for optimisation of qPCR assays, particularly in the use of cell lines. Certain transcripts may be overexpressed in immortalised cell lines, whilst in others, they may be underexpressed or lacking altogether.

2.1.3.5 RNA Quality

The accuracy of gene expression assays is critically influenced by the quantity and quality of the RNA extracted prior to real-time PCR (Fleige and Pfaffl, 2006). The quantification and integrity assessment of extracted RNA is an essential practice to ensure the accuracy and reproducibility of a qPCR-based experiment or any other RNA-based analysis such as RNA-seq (Fleige and Pfaffl, 2006). Low-quality RNA has a high probability of compromising the accuracy of potential downstream applications.

Several steps should be taken prior to downstream analyses to ensure that the RNA is of sufficiently quality for accurate qPCR assays. Purified RNA should adhere to several criteria. Firstly, the RNA preparation should be free from contaminating cellular artefacts such as genomic DNA and proteins. Total RNA should also be free of reagents which were used in the initial isolation such as ethanol or guanidine thiocyanate which can inhibit reverse transcriptase or downstream qPCR. The most important aspect for the extraction of high-quality RNA is to avoid RNase contamination. Contamination will result in the degradation of RNA and loss of ability to accurately assess sensitive gene expression profiles. RNase contamination also hampers the ability of the RNA to be stored long term. Fortunately, commercially available RNase inhibitors are available for the efficient removal of RNases from lab surfaces and pipettes. The use RNase-free tubes, pipette tips, and other consumable reagents is an essential step for any RNA work.

Analysis and quantification of total RNA can be done in one of three ways. Traditionally, the gold standard for the detection of RNA quality was to run extracted RNA on a 3-(N-morpholino) propanesulfonic acid (MOPS) gel via electrophoresis. Visualisation of two distinct bands, 28S (±4,600 bases) and 18S (±1,800 bases) with limited smearing, is indicative of high-quality RNA. This has been improved upon using modern microfluidic technology in instruments such as Agilent's Bioanalyzer which allows for the assessment of both the quantity of RNA and its associated quality via an RNA integrity number (RIN) from 1-10 (where 10 is RNA of the highest quality). For qPCR and RNA-Seq, RIN values should always be greater than 7. A less accurate, but faster method of RNA integrity assessment is via spectrophotometry. NanoDrop technology allows for the assessment of RNA quality and quantity via this method. Quality is assessed as a ratio of absorbance at 260 nm and 280 nm, or 230 nm. High quality, contaminate-free RNA should have an A260/A280 (or A230) ratio in the range 1.8-2.0 prior to cDNA synthesis and qPCR.

2.2 Methods and Materials

2.2.1 Cells and Reagents

2.2.1.1 THP-1 Cells

2.2.1.1.1 Background of THP-1 Cell Line

THP-1 cells are a monocytic cell line acquired from a one-year-old infant suffering from acute monocytic leukaemia and are available commercially from the American Type Culture Collection (ATCC) (ATCC, 2015). Cells reside in suspension and are phagocytic whilst lacking surface and cytoplasmic IgG (Chanput et al., 2014). In the undifferentiated state, THP-1 cells are weakly stimulated via the application of toll-like receptor agonists, however, this situation changes upon their activation to macrophages (Chanput et al., 2014). Such differentiation is achieved via the application of phorbol 12-myristate 12 acetate (PMA) (Park et al., 2007). Application of PMA for cellular activation is terminal (irreversible), meaning that THP-1 cells will no longer undergo mitosis and will adhere and metabolise more slowly in a manner characteristic to macrophages (Chanput et al., 2014). For this study, THP-1 cells were evaluated as monocytes.

2.2.1.1.2 Maintenance of Cell Culture

THP-1 cells were acquired (ATCC) and maintained in ATCC formulated RPMI-1640 medium supplemented with 2-mercaptoethanol (0.5 mM) (Sigma-Aldrich), fetal bovine serum (10%) (Merck), Non-Essential Amino Acids (NEAA) (1%) (Gibco®), HEPES (10%), L-glutamine (10%) (Lonza) and sodium pyruvate (1%) (Gibco®). The cells were incubated horizontally in a 5% CO_2 environment at 37° C and maintained at a cell concentration of ±1.0 x 10^6 cells/ml. Media was substituted correcting for the right cell concentration every 3 days. The cells were expanded until an adequately high number was generated at which time they were frozen down at a concentration of 5x10^6 cells/mL and stored in liquid nitrogen as per 2.2.1.1.3 below. To control for potential additive deleterious effects which may arise due to the long culture of THP-1 cells *in vitro,* all THP-1 cells required for downstream optimisation and stimulation experiments were used for optimisation and stimulation experiments at a passage of 20 or below.

2.2.1.1.3 Freezing & Cryopreservation

Freezing of THP-1 cells was implemented in accordance with the protocol set out by the ATCC. Briefly, THP-1 cells were spun down at 15000 rpm for 10 min, resuspended in freezing medium consisting of 40% standard culture media (see 2.1.1.2 above), 10 % dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 50 % fetal bovine serum (FBS). Cells were then placed in a 2 ml cryogenic vial (Corning). Cryovials were consigned to a stratacooler (Agilent Technologies) and placed in a -80° C freezer overnight. The following day, cryovials were immediately transferred to gas-phase liquid nitrogen storage.

2.2.1.1.4 Thawing of THP-1 cells

Thawing of THP-1 cells was done in accordance with the protocol set out by the ATCC. Frozen THP-1 cells were retrieved from gas-phase liquid nitrogen storage and transferred to a 37° C water bath and agitated gently. Samples were thawed rapidly until a single ice chip remained at which point the cryovial was decontaminated using 70% ethanol. Samples were then transferred drop-wise into 9 ml of complete culture media (see 2.1.1.2) and spun down at 1500 rpm for 7 min. The pellet was resuspended in complete culture medium and incubated in accordance with the details mentioned in 2.1.1.2 above.

2.2.2 RNA Isolation and cDNA Synthesis

2.2.2.1 Principle of Methods

Any investigation into gene expression is fundamentally based on an understanding of the central dogma of biology. The principle underlying the dogma: transcription of a nucleic acid sequence information (gDNA -> mRNA) and the subsequent translation of that sequence into a protein (mRNA -> protein). The process involves three major biopolymers: DNA, RNA and proteins. DNA is unwound by an enzyme (DNA helicase) that then allows for RNA polymerase to start transcribing a strand of the messenger, or mRNA from the DNA template. Once transcribed the non-coding introns of the mRNA are then spliced out of the mRNA strand, leaving an mRNA composed of exons alone. This is called post-transcriptional modification, which gives rise to mature mRNA. These strands are then translated by ribosomes into chains of amino

acids to form proteins. These proteins then undergo conformation change in a process termed post-translational modification to form mature proteins.

During gene expression analyses nucleated cells are activated or have some treatment imposed on them for a certain period, after which the cells are then lysed/homogenised. The mRNA present represents gene expression and may have a non-linear relationship to levels of proteins (which can be studied using proteomics). RNA is then isolated, purified and quantified. Complementary DNA (cDNA) refers to the single strand of DNA which is artificially transcribed and amplified from the mRNA utilising primers, reverse transcriptase (RT) and deoxyribonucleotide triphosphate (dNTPs). Such amplification is achieved using varying thermal cycles which facilitate the binding of primers, the activity of reverse transcriptase and melting of the cDNA from the mRNA template. The synthesis of cDNA continues until all reaction reagents are used up at which point the concentration of cDNA plateaus out. The cDNA can then be analysed via PCR and qPCR using gene specific primers to assess relative gene expression.

2.2.2.2 RNA Isolation, QC and Reverse Transcription Procedure

For all isolations, cells (>1x10^6 cells/ml) were pelleted and resuspended in Buffer RLT Plus and homogenised (Qiagen, RNeasy® Plus Mini Kit cat. No. 74104). RNA lysates were transferred to gDNA Eliminator spin column and centrifuged at 9000 x g for 30 sec. The supernatant was removed to a new RNeasy spin column and centrifuged at 9000 x g for 15 sec. Flow through was then discarded. RW1 buffer was added and the tube was spun again at 9000 x g for 15 sec. Flow through was then discarded. RPE buffer was added and the tube was spun again at 9000 x g for 15 sec. Flow through was then discarded. RPE buffer was added and the tube was spun again at 9000 x g for 2 min. RNase-free water was added to the spin column and spun at 9000 x g rpm for 1 min to elute RNA. Eluted RNA was then quantified via Nanodrop. The cDNA templates were reverse transcribed from isolated RNA using iScript™ cDNA Synthesis Kit (BioRad, cat. No. 1708891). RNA and cDNA concentration was then analysed via Nanodrop (Nanodrop Lite Spectrophotometer, ThermoScientific).

2.2.3 Primers and Real Time PCR

The PCR primers used for cathelicidin (CAMP), cytochrome P450C24 (CYP24a1), cytochrome P450 family 27 subfamily B member 1 (CYP27b1), β-Defensin 2 (DEFB4) and vitamin D receptor (VDR) real-time quantitative PCR were validated in our laboratory using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (see Appendix 2 and 3) and Primer 3 Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to ensure that all primers fulfilled the criteria required for an optimal primer for each qPCR target gene (see Appendix 1 and discussion in 2.3.1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC) and β-actin (ACTB) were utilised as reference genes for normalisation of data. Amplified product for each set of primers was subjected to standard PCR and run on a 2.5% agarose gel to determine whether the desired product was being amplified as well as to optimise the PCR conditions (annealing temperature and extension time) before moving on to real-time PCR optimisation.

The qPCR assays for CAMP, CYP24a1, CYP27b1, DEFB4, VDR, UBC, GAPDH and ACTB were performed on a LightCycler® 480 Real-Time PCR System (Roche). Each PCR reaction comprised of 0.0625 – 0.25 pmol/µL of each primer, 5 µL SYBR Green I Master mix (2X) (Roche), 1 µL cDNA and water to 10 µL. Reactions were run in duplicate on a Roche LightCycler® 480 version 1.5 with one cycle at 95 ˚C (10 min), followed by 45 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72° for 30 seconds. Detection of the fluorescent products was carried out at the end of the 72 ˚C extension period. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and 2.5% agarose gel electrophoresis. Serial dilutions of cDNA from total RNA were performed for each target gene. These served as standard curves for quantitative analysis. Controls without any added RNA (No template controls) were tested and demonstrated the absence of amplification to ensure experimental validity.

Table 1: Final Optimized Primers Sequences and Associated Information

Gene Code	*Ref Seq.*	*Product Size (bp)*	*[Primer] (pM)*	*Forward Primer (5' – 3')*	*Reverse Primer (5' – 3')*
CAMP	NM_004345.4	59	0.250	CCAGGACGACACAGCAGTCA	CTTCACCAGCCCGTCCTTC
VDR	NM_000376.2	135	0.250	AGGCTGCAAAGGCTTCTTC	ATGTCCACACAGCGTTTGAG
CYP24a1	NM_000782.4	61	0.250	CGCAGCGGCTGGAGAT	CCGTAGCCTTCTTTGCGGTA
CYP27b1	NM_000785	233	0.250	TCCATCCTGGGAAATGTGACA	ACAGGGTACAGTCTTAGCACTT
DEFB4	NM_004942.3	75	0.250	GGTGTTTTTGGTGGTATAGGCG	AGGGCAAAAGACTGGATGACA
GAPDH	NM_002046.5	225	0.0625	AAGGTCGGAGTCAACGGATT	CTCCTGGAAGATGGTGATGG
UBC	NM_021009.6	202	0.50	TTTCCAGAGAGCGGAACAG	TCACGAAGATCTGCATTGTCAAG
ACTB	NM_001101.3	121	0.50	GATCAAGATCATTGCTCCTCCTG	GCCGGACTCGTCATACTCC

2.3 Results

Each gene was taken through all optimisation steps explained above. In order to avoid repetition, the following section will focus on the troubleshooting of the problems that occurred during qPCR assay design and optimisation and a discussion of the lessons learned and issues addressed in each area of optimisation.

2.3.1 Bioinformatic Validation of Primers

A literature search of published qPCR-based research was conducted to acquire published primer pairs for the transcripts of interest. Optimal primers were then shortlisted to satisfy a number of conditions. They should be approximately 19-25 base pairs in length and amplify a product no bigger than 400 base pairs to ensure similar melting temperatures and specific amplification of product. Longer amplicons (>400bp) have a tendency toward forming nonspecific secondary peaks that hamper the accuracy of the assay. They should be designed to reduce the chance of secondary structure (hairpins, self-dimer etc.) formation as these structures may inhibit the binding of the primers to the target sequence or result in no specific peaks. The GC content of each primer should be in the range of 20-70 % and each member of the pair should have similar melting temperatures to ensure efficient binding and dissociation of the primers to the target cDNA sequence. For gene expression, it is important that the primers should account for all known isoforms or variants of the target sequence. Exon-exon spanning primers are desirable to reduce the chance of genomic DNA contamination, and if present, allow for its easy identification (Dieffenbach et al., 1993).

Each primer pair identified from the literature was assessed using NCBI's Primer-BLAST to ensure that a) the primers were detected by the database and b) the primers were exon-exon boundary spanning (see Appendix 2). The primer pair was then BLASTed against the human genome to assess the specificity of the primer to a specific transcript (Figure 19). Among those identified in the literature, primer pairs for each transcript of interest were selected for further consideration based on their specificity and as well as the ability to amplify all known variants via the amplification of a single product.

Enter accession, gi, or FASTA sequence (A refseq record is preferred) Clear

NM_000785

Or, upload FASTA file   Choose file No file chosen

Primer Parameters

Use my own forward primer (5'->3' on plus strand)   TGAGACTGCTGGAGCTGAGA

Use my own reverse primer (5'->3' on minus strand)   AGCCTTCCAAAGATCCCCTA

Exon/intron selection   A refseq mRNA sequence as PCR template input is required for options in the section

Exon junction span   Primer must span an exon-exon junction

Exon junction match   Exon at 5' side 7   Exon at 3' side 4

Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction

Intron inclusion   Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range   Min 1000   Max 1000000

Primer Pair Specificity Checking Parameters

Specificity check   Enable search for primer pairs specific to the intended PCR template

Search mode   Automatic

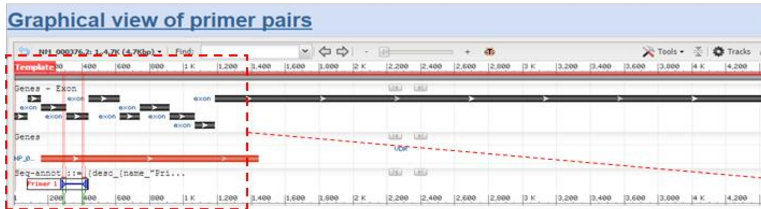
Database   Refseq mRNA

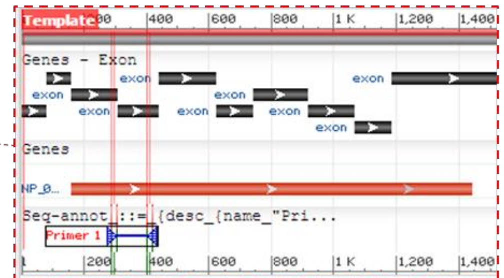
Exclusion   Exclude predicted Refseq transcripts (accession with XM, XR prefix)

Organism   Homo sapiens

Enter an organism name (or organism group name such as enterobacteriaceae, rod

Graphical view of primer pairs





Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGGCTGCAAAGGCTTCTTC	Plus	19	286	304	58.35	52.63	5.00	3.00
Reverse primer	ATGTCCACACAGCGTTTGAG	Minus	20	420	401	58.77	50.00	4.00	2.00
Product length	135								

Products on intended target

>NM_000376.2 Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), transcript variant 1, mRNA

```
product length = 135
Forward primer  1    AGGCTGCAAAGGCTTCTTC  19
Template        286  ...................  304

Reverse primer  1    ATGTCCACACAGCGTTTGAG  20
Template        420  ....................  401
```

Products on potentially unintended templates

>XM_006719587.3 PREDICTED: Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), transcript variant X2, mRNA

```
product length = 135
Forward primer  1    AGGCTGCAAAGGCTTCTTC  19
Template        203  ...................  221

Reverse primer  1    ATGTCCACACAGCGTTTGAG  20
Template        337  ....................  318
```

>XM_011538720.2 PREDICTED: Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), transcript variant X1, mRNA

```
product length = 135
Forward primer  1    AGGCTGCAAAGGCTTCTTC  19
Template        318  ...................  336

Reverse primer  1    ATGTCCACACAGCGTTTGAG  20
Template        452  ....................  433
```

>NM_001017536.1 Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), transcript variant 3, mRNA

```
product length = 135
Forward primer  1    AGGCTGCAAAGGCTTCTTC  19
Template        677  ...................  695

Reverse primer  1    ATGTCCACACAGCGTTTGAG  20
Template        811  ....................  792
```

Figure 19: Bioinformatic Validation of Primers for Vitamin D Receptor using NCBI's Primer-BLAST. The online analysis allows for the detection of potential off-target amplicons, primer binding regions, specificity and GC content of both sense and antisense primer.

Prospective primer pairs were then assessed using the Primer 3 Plus online tool (Figure 20) to calculate the probability of secondary structure formation between the given primers, the GC content and 3' end stability.

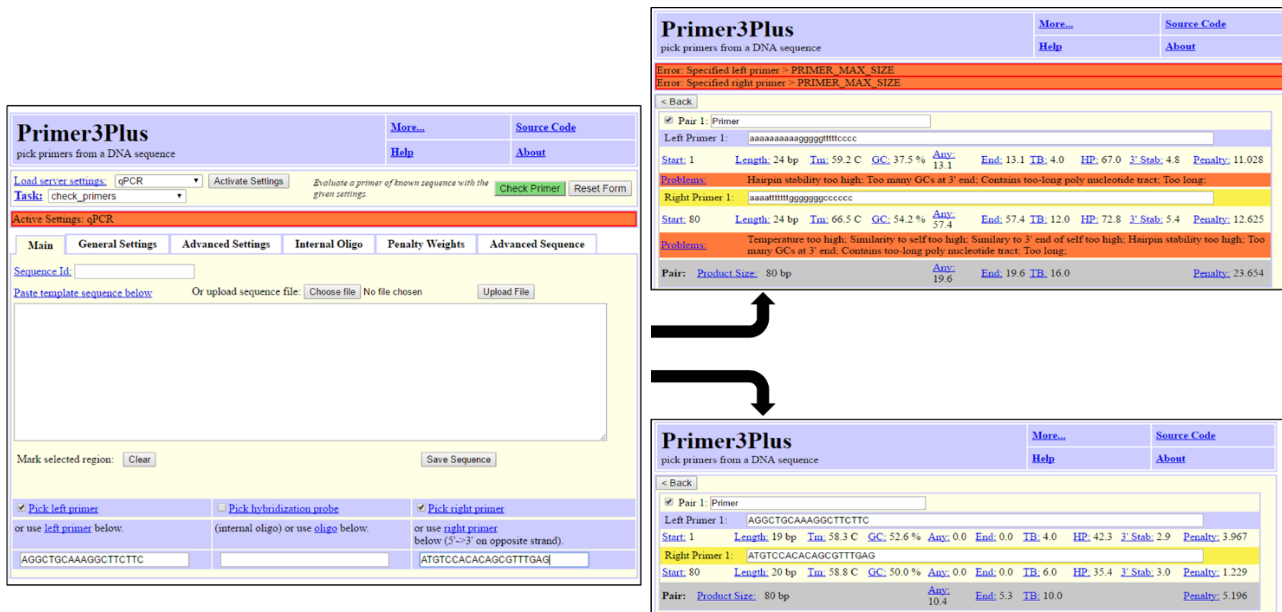


Figure 20: Primer 3 Plus Analysis for Secondary Structures, GC Content and 3' Stability for Vitamin D Receptor (Bottom Right) and an Example of an Inappropriate Primer Pair (Top Right)

Primer sequences which failed to pass both of the online analyses were discarded, whilst primers which passed were ordered, synthesised and tested in-house. All primer pairs listed in Table 1 were tested and found to satisfy all the bioinformatic requirements for an optimal primer. Having been bioinformatically validated, the primers were then tested on the THP-1 cell line.

2.3.1 Validation of Genes of Interest by Agarose Gel Electrophoresis

To further validate the specificity of the primer sets designed for CAMP, VDR, CYP24a1, CYP27b1 and DEFB4, the amplified PCR products were subjected to 2.5% agarose gel stained with SYBR Safe. Each product demonstrated a single amplicon (Figure 21). The amplified PCR product sizes were all in agreement with the bioinformatically predicted values (see Table 1).

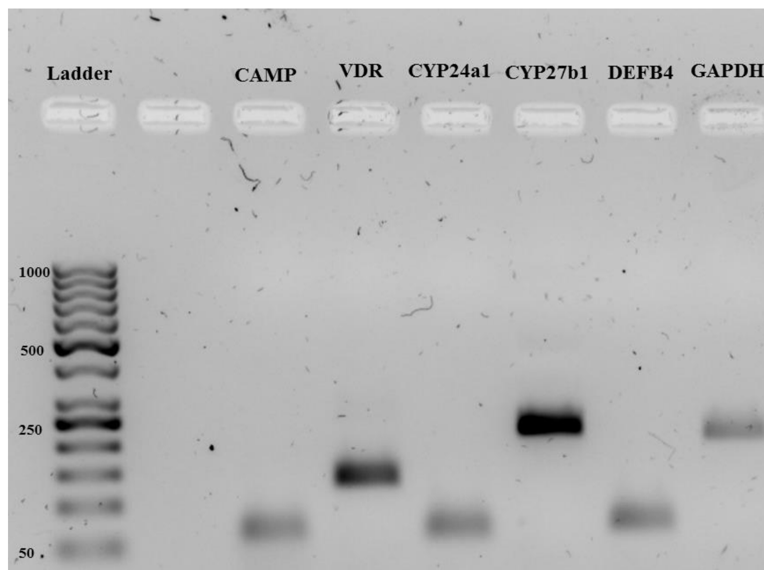


Figure 21: Gel Validation of Amplified Genes of Interest Product Size via Gel Electrophoresis. The size of each band correlated well with the predicted product length.

2.3.2 Optimisation of qPCR Conditions to Remove Primer-Dimer

Initial experiments with a newly designed primer pair were conducted using the primers at a final concentration of 0.50 pM. When real-time experiment PCR melt curves showed the presence of primer-dimers (secondary peak which melts at a lower temperature relative to the specific peak or a peak present in the no template control), the final primer concentration in the reaction was diluted two-fold until the primer-dimer was no longer visible on the melt curve analysis. Figure 22 illustrates this for the GAPDH gene amplification primer pair; here the final concentration of the forward and reverse primers were

sequentially reduced until the secondary peak at 76-78°C (indicative of primer-dimer in both the sample and no template control) was completely eliminated at a concentration of 0.0625 pM.

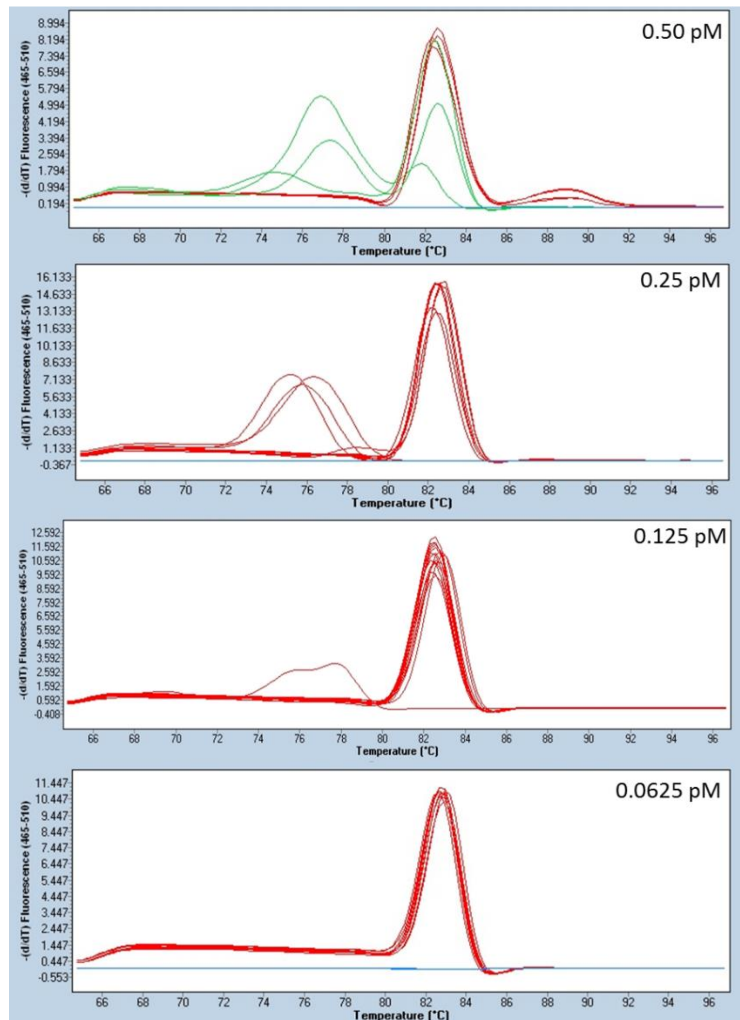


Figure 22: Elimination of Primer-Dimer in for the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) qPCR via serial dilution of Primers.

2.3.3 Optimisation of qPCR Conditions to Remove Non-Specific Amplification

2.3.3.1 Genomic DNA Contamination of template cDNA

Non-specific amplification is the undesirable amplification of an unintended secondary product during the PCR. This is generally detectable by observation of a separate peak during melt curve analysis.

Primers were designed such that only specific amplicons would be amplified in the absence of contaminating DNA. In the presence of genomic DNA contamination, the exon-exon spanning design of the primers allowed for its easy detection since the unintentional product (intron-containing) was longer and melted at a higher temperature relative to that of the intended product.

This optimisation is illustrated for the CAMP gene. Melt curve analysis was carried out after real-time PCR of CAMP from THP-1 samples. The specific product melted at approximately 81° C and a secondary peak was observed to melt at approximately 87° (Figure 24B). The no template control (NTC) was devoid of any amplification, implying that the secondary peak was likely the result of genomic DNA contamination of the template cDNA rather than primer dimer or DNA contamination. To test this idea, isolated RNA, stored from the same experiment had been at -80° C was re-concentrated via ethanol precipitation and subjected to additional DNase treatment.

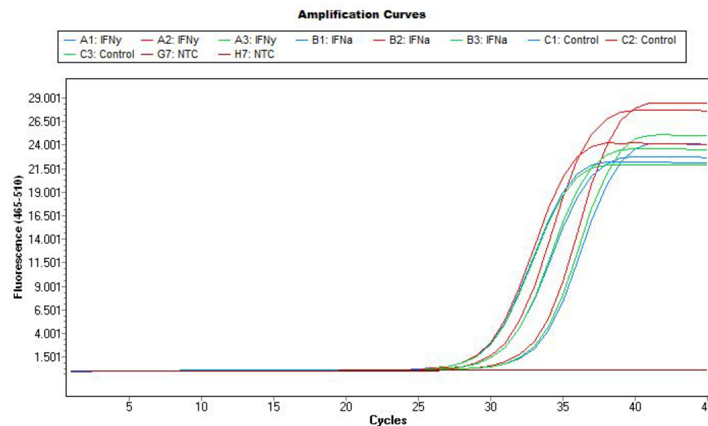


Figure 23: Amplification Curve of Cathelicidin (CAMP)

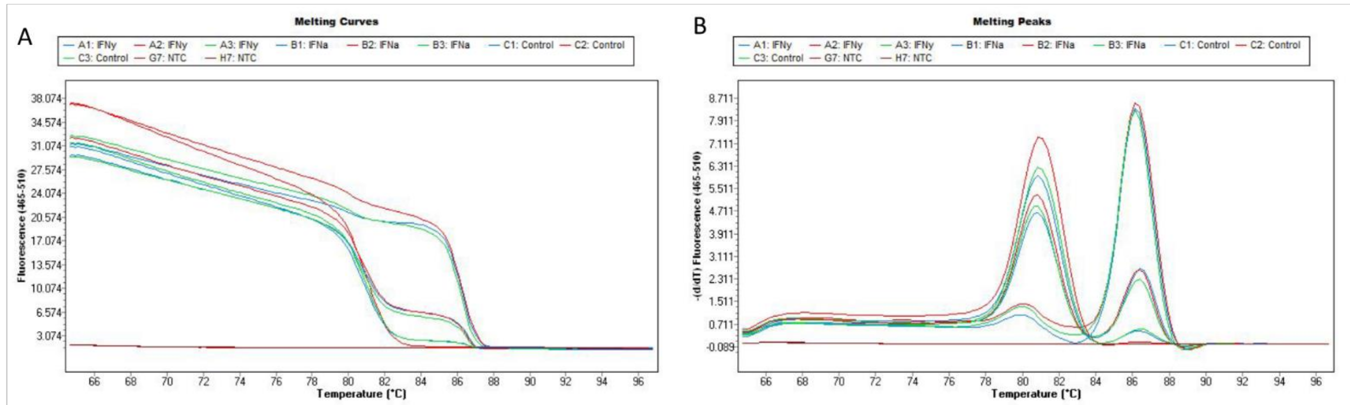


Figure 24: Melt Curve Analyses of CAMP showing A) the Melting Curves and B) the Associated Melting Peaks. Non-specific amplification as a result of genomic DNA contamination can be observed at a melting temperature of approximately 86°C.

After the additional DNase treatment, the samples were reverse transcribed and used for real-time PCR. The resulting melt curve analysis showed that the additional DNase treatment completely eliminated the secondary product (Figure 26B). The primer pairs specific for CAMP were found to be particularly sensitive to any gDNA contamination.

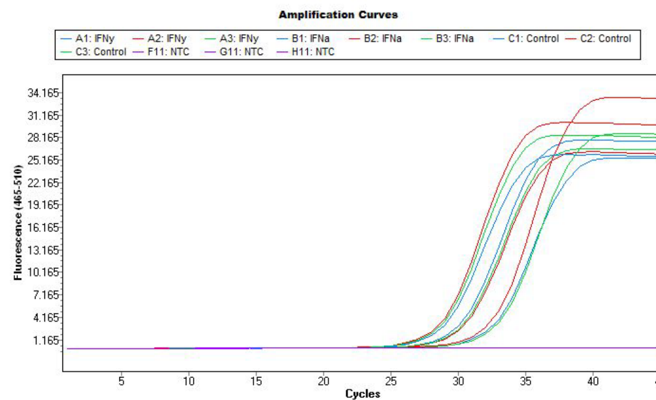


Figure 25: Amplification Curves of CAMP Post Additional DNase Treatment

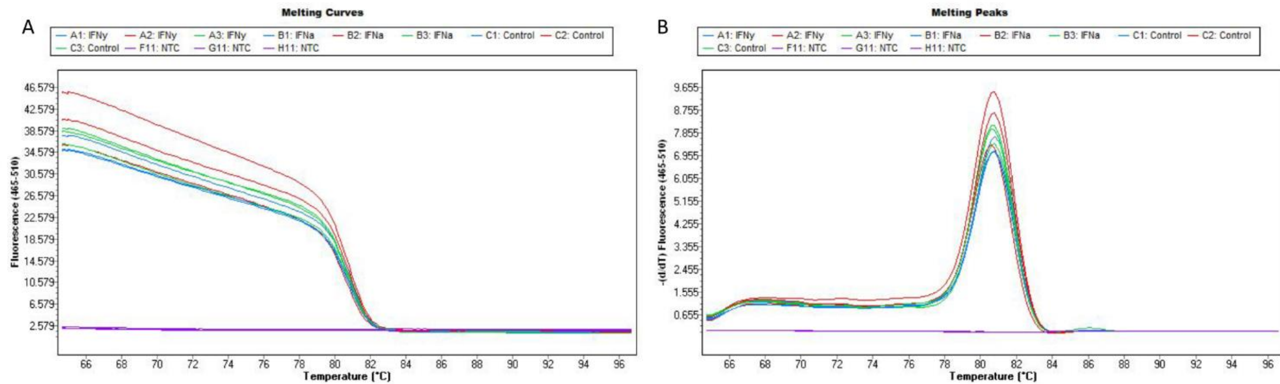


Figure 26: Melt Curve Analyses of CAMP showing A) the Melting Curve and B) the Associated Melting Peaks Post Additional DNase Treatment showing a single specific product being amplified.

Genomic DNA contamination of total RNA also interfered with the ability of the NanoDrop to accurately quantify RNA post-nucleic acid extraction. As such, this skewed the reaction concentration of DNase used during the removal of DNA from the total RNA preparation. A higher reaction volume of 1.5 times the recommended volume of DNase was used per sample to overcome this particular issue and was successfully used in all RNA extractions going forward.

2.3.4 Assessment of RNA Quality

The quality of the purified, DNase treated RNA was assessed for use in downstream real-time PCR applications using the Agilent 2100 Bioanalyser. The quality of the RNA used in any gene expression assay is the single most important aspect of the assay. Poor quality RNA can affect the point at which a real-time PCR machine can detect signal by significantly delaying amplification. If the objective of the assay is to look for a low copy transcripts RNA quality becomes even more important as these sensitive transcripts may be lost due to damaged RNA. An RNA integrity number (RIN) value of 7 or above is adequate for real-time applications.

Purified, DNase-treated RNA from lysed THP-1 cells yielded a RIN value of 8 indicating high-quality nucleic acid which was of sufficient quality to be reverse transcribed into cDNA and utilised for

downstream qPCR (Figure 27). A high-quality RNA is illustrated by two peaks at 2000 and 4000 nucleotides.

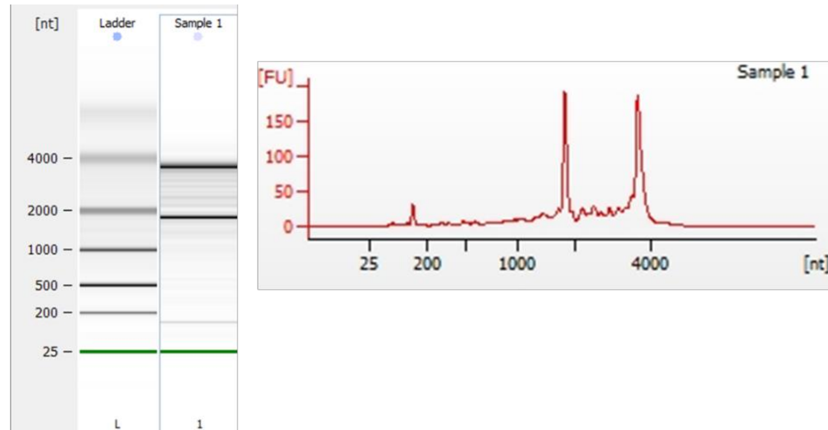


Figure 27: Output from Agilent Bioanalyser 2100 Showing High-Quality RNA (RIN=8) Extracted using the RNeasy Plus Mini Kit in Conjunction with an Additional DNase Treatment.

2.3.5 Formation of Standard Curves for Relative Analyses

Complementary DNA (cDNA) synthesised from 1 µg of RNA was serially diluted and subjected to qPCR to create an individual standard curve for each gene of interest. The primary objective of this was to determine the efficiency of the qPCR assay per gene which is required to accurately quantify gene expression relative to the reference gene (GAPDH) using the Pfaffl equation (Pfaffl, 2001);

$$\boldsymbol{R = \frac{Effciency(target)^{Ct(Target,\ Untreated)-Ct(Target,\ Treated)}}{Efficiency(Reference)^{Ct(Target,\ Untreated)-Ct(Target,\ Treated)}}}$$

The Pfaffl equation is used in instances where the qPCR reaction efficiencies between the gene of interest and the reference gene are not similar or equal to 2 (100% efficient). For all genes of interest, the reaction efficiencies were optimised to be greater or equal to 85% and have an error less than 0.2 (Table 2.).

Table 2: Summary of Optimized Standard Curves for All Genes of Interest

Gene Code	*Efficiency (%)*	*Slope*	*Error*
CAMP	92	-3.521	0.0428
VDR	89	-3.614	0.00427
CYP24a1	92	-3.534	0.00415
CYP27b1	95	-3.443	0.00609
DEFB4	94	-3.475	0.106
GAPDH	86	-3.704	0.0228
UBC	92	-3.528	0.0370
ACTB	94	-3.487	0.276

2.3.6 Lack of Reliable Expression of DEFB4 in THP-1 Cells

Beta-defensin 2 (DEFB4) primers were bioinformatically validated using primer-BLAST. However, qPCR analysis of DEFB4 in THP-1 cell line revealed no difference between the no template control and cDNA synthesised from THP-1 cells (Figure 28 A-C). Complementary DNA synthesised from a reference RNA library (Human Reference RNA, Agilent), composed of 10 differing human tissues, was utilised to test the DEFB4-specific primers under the standard optimised qPCR cycling conditions. In contrast to the THP-1 cell line, the specific product was amplified and detected from the reference cDNA libraries synthesises from comparable total RNA (Figure 28 D-F). Like DEFB4, CYP24a1 also was also undetectable in THP-1 cells - but was reproducibly detectable within the reference cDNA library (Data not shown). Further, specific amplification of both transcripts was again reproducibly observed in patient samples during stimulation experiments (see Chapter 3).

From this, we concluded that the THP-1 cells which were purchased from the ATCC and utilised for the *in vitro* optimisation of these qPCR assays were either lacking DEFB4 and CYP24a1 or the expression of these genes was below the limit of detection in this cell line in contrast to primary cells.

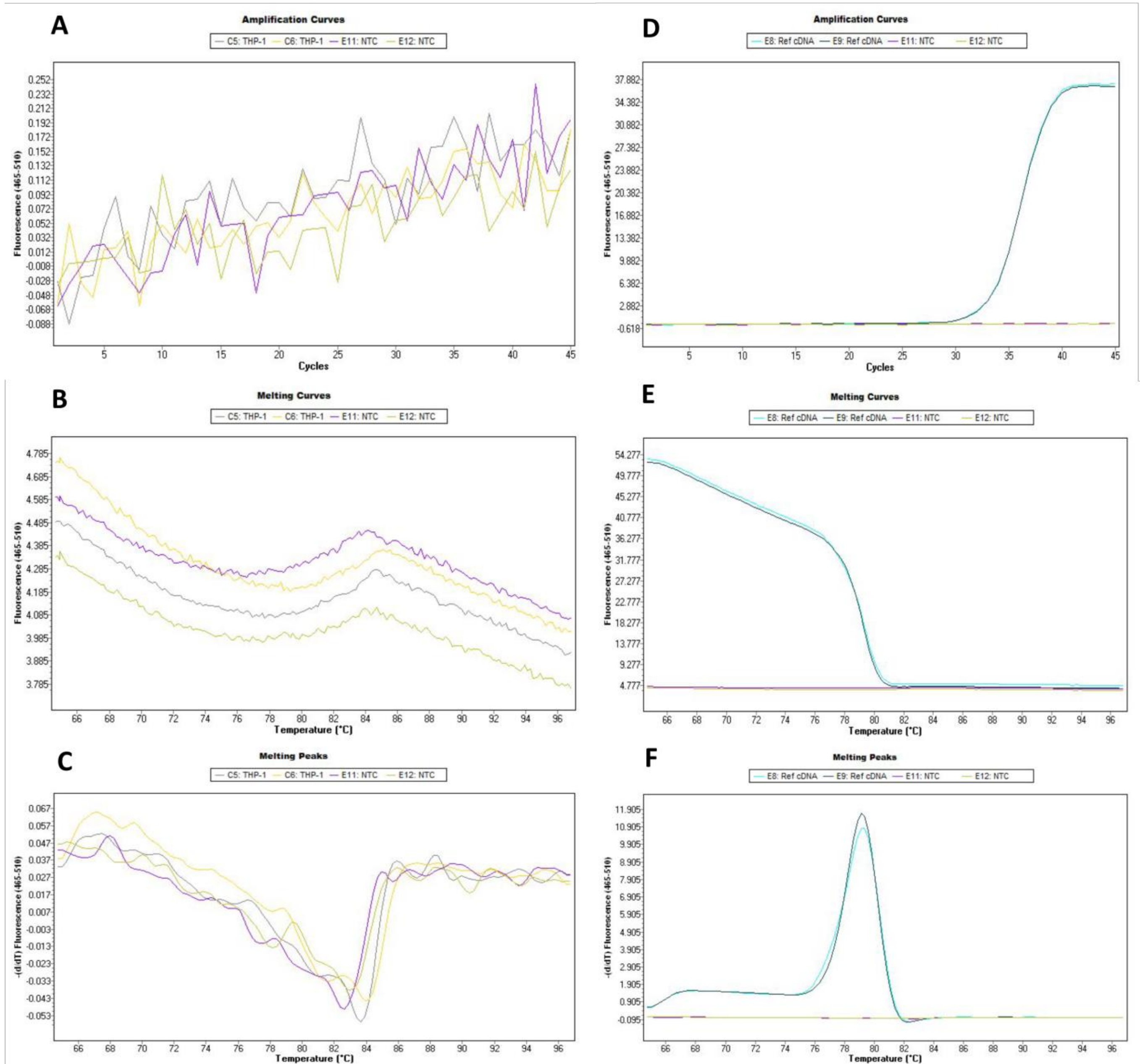


Figure 28: Detection of DEFB4 in THP-1 Cells vs. a Reference cDNA Library. A) Amplification curves of DEFB4 in THP-1 cells. B) Melting Curves of DEFB4 in THP-1 cells. C) Melting peaks of DEFB4 in THP-1 cells. D) Amplification curves of DEFB4 in a reference cDNA library. E) Melting Curves of DEFB4 in a reference cDNA library. F) Melting peaks of DEFB4 in a reference cDNA library.

2.3.7 Validation of Reference Genes

The accuracy of relative gene expression experiments using real-time PCR is highly reliant on the simultaneous measurement of a stably expressed endogenous control gene that is not affected by an experimental treatment. Measurement of such a gene allows any variation observed between treatments to be analysed after normalisation to cell number. Although certain "housekeeping" genes are generally selected for use as a reference, it is best practice to experimentally determine whether expression of a selected reference gene is stable under the experimental conditions specific to a set of experiments.

To test the stability of expression under stimulation conditions, we tested three potential reference genes (Figure 29). Primers for hypoxanthine-guanine phosphoribosyltransferase (HPRT), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S were optimised (Figure 28). THP-1 cells (2×10^6) were stimulated with recombinant cytokines (IFN-γ or IL-10) or were left unstimulated. After cell lysis, 0.5 µg total RNA from each condition was reverse transcribed and used for qPCR analysis. For each of the three genes, there was relatively little variation in the expression of each gene across the three conditions. Interestingly, the pattern of slight variation observed across conditions was common to all three reference gene candidates which we concluded meant that differences in cell number between conditions, rather than condition-specific induction of each of the transcripts, accounted for the differences. Despite efforts to keep cell numbers between the experimental conditions equivalent for this experiment, this artefact could have occurred during cell culture, nucleic acid extraction/purification, or reverse transcription of mRNA to cDNA. As all three potential reference genes behaved equivalently and showed no evidence of modulation by the cytokine stimulations, we decided to move forward with GAPDH as an appropriate reference gene for use in relative quantification of transcripts of interest.

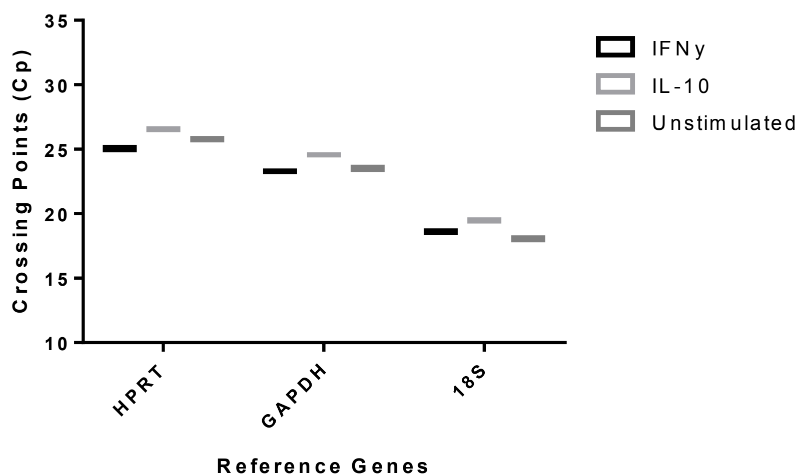


Figure 29: Reference Gene Validation Experiment. HPRT, GAPDH and 18S all found to be suitable reference gene candidates for downstream relative qPCR gene expression assays.

2.4 Discussion and Conclusion

Even in the face of newer technologies such as RNA-seq real-time PCR remains the gold standard for gene expression assays in molecular studies. In this study, real-time PCR assays for the reliable and reproducible detection of CAMP, VDR, CYP24a1, CYP27b1 and DEFB4 were optimised for relative analysis using the Light Cycler 480. SYBR Green was selected over hybridization probes as the chosen method of quantitative chemistry for this thesis due to its ease of design, economical attributes, simple validation of amplified product (via melt curves) as well as the ability to better optimise a real-time PCR reaction per individual genes. However, the trade-off of this detection chemistry is the inability to design multiplex experiments.

Primers were bioinformatically validated *in silico* and tested for the THP-1 cell line to test for reliable reproducibility of gene expression. All genes were optimised to work under the following qPCR conditions; pre-amplification for 7 minutes at 95° C, followed by 45 cycles of denaturation at 95° C for 30 seconds, annealing of the primers at 60° C for 30 seconds and elongation of the product at 72° C for 30

seconds. All amplified transcript product sizes were confirmed via gel electrophoresis and found to be the expected size.

Detectable, accurate standard curves which adhere to the MIQE guidelines were constructed for CAMP, VDR, and CYP27b1 using the THP-1 cell line. In contrast, such standard curves could not be constructed for CYP24a1 and DEFB4 using the THP-1 cell line. In contrast to previously published studies, THP-1 cells cultured *in vitro* in our laboratory seemed to express low levels of these transcripts that were below the limit of detection even at high cell numbers (Adams et al., 2009, Gottfried et al., 2006, Heulens et al., 2016). However, as will be presented in Chapter 3, DEFB4 and CYP24a1 were accurately detected in human monocytes, alveolar macrophages and bulk PBMCs suggesting that the "problem" for these two transcripts was specific to THP-1 cells. In order to overcome this problem, real-time PCR assays for both of these genes were optimised using pooled reference mRNA which was reverse transcribed to cDNA. The lack, or minimal expression, of these transcripts in THP-1 cells, may be due to several potential factors. Firstly, the culture of THP-1 cells in vitamin D-deficient media may have had an inhibitory effect on the ability of these monocytic cells to transcribe mRNA relevant to this particular pathway, this will be fully discussed in Chapter 3 of this manuscript. Alternatively, the immortalised nature of the cell line may have contributed to this phenomenon. It is possible for cells which are constantly dividing for a long period to acquire mutations or short nucleotide polymorphisms which may modulate the cells ability to express particular transcripts. Lastly, the expression of these particular transcripts may only be facilitated through the adequate activation of the pathway via an appropriate TLR-agonist association. A potential limitation of this assay is the phenomenon of transcriptional data not necessarily correlating with protein data (Gry et al., 2009). Although this is beyond the scope of this study, the next step would be to conduct quantitative protein assays such as Luminex, ELISA or Western blot for the validation of transcriptomic data and facilitation of stronger conclusions.

In conclusion, the real-time PCR assays for the detection of antimicrobial peptides and upstream modulators that have been optimised and presented here will allow for the opportunity to answer a multitude

of questions for future *in vitro* and *ex vivo* studies in our laboratory. Using the methodology described in this chapter, additional qPCR assays can be developed to validate novel genes of interest which may be identified using unbiased hypothesis-generating technologies such as RNA-seq. I found that efficient elimination of genomic DNA was achieved using a gDNA elimination column (Qiagen) in conjunction with an additional DNase treatment provided high quality (RIN=8) RNA that was appropriate for use in downstream qPCR assays. Interestingly, I found that that CYP24a1 and DEFB4 expression in unstimulated THP-1 cells was either inhibited or lacking entirely. In the data presented here, I have optimised a one-plate real-time PCR assay for the accurate and reliable detection of CAMP, VDR, CYP24a1, CYP27b1 and DEFB4; this work provided the methodological background for the experiments presented in Chapter 3 and will facilitate future experiments that require the relative quantification of these transcripts during bacterial/viral infection of innate immune cells.

3 CHAPTER THREE: Effect of Type I/II Interferon Stimulation on the Expression of Transcripts of Interest in Healthy Patient CD14+ Cells and the THP-1 Monocytic Cell Line

3.1 Brief Introduction

Cell lines have been successfully used as biologically relevant proxies of primary human cells/tissues in medical research for many years. They provide a platform for the study of human cells without the ethical and practical constraints that restrict the acquisition and use of primary cells/tissues from human donors (Chanput et al., 2014). There is, however, a debate around the appropriate use of cell lines vs. primary cells for the optimisation of assays for the investigation of specific biological questions that have clinical implications. Certainly, results that have been achieved in cell lines should be validated in primary human tissues. The THP-1 is a monocytic cell line acquired from a one-year-old male infant suffering from acute monocytic leukaemia which was first characterised in 1980 (Chanput et al., 2014, Tsuchiya et al., 1980). This cell line has been successfully used in many studies over the years as a model

system for elucidating monocytic and macrophage biochemical pathways, bacterial infection/phagocytosis assays, signalling pathways and the transport of nutrients, proteins and drugs (Chanput et al., 2014).

The use of THP-1 cells has several advantages; Firstly, THP-1 cells allow for greater reproducibility between multiple experiments due to their homogenous phenotype and genotype, thus reducing variation commonly observed from patient-to-patient when using primary cells. Secondly, they recover well from cryopreservation and can remain in culture for up to three months (passage 25) without deleterious changes in cell activity or loss of sensitivity to an antigen or drug (Chanput et al., 2014). The cells have been reported to double every 35-50 hours making them readily available for research purposes in a short duration of time. Conversely, the acquisition of buffy coats for large scale primary monocyte work is more time intensive or limited in many research settings.

THP-1 cells' ease of use is, however, offset by some inherent limitations. *In vitro* differentiation of THP-1 cells to a more macrophage-like state facilitates the over-expression of certain genes; the induction of this transcriptional program is thought to perhaps mask the subtle, though significant, effects of certain stimulants, ligands and agonists. (Schildberger et al., 2013, Bruckmeier et al., 2012, Hijiya et al., 2002, Park et al., 2007). The *in vitro* culture of THP-1 cells under controlled conditions (relative to their natural environment within the host) is also hypothesised to have an effect on the phenotype and gene expression profile of THP-1 cells, due to lack of adequate cell signalling molecules or media deficient in some endogenous compounds required for "normal" functioning and expression (Schildberger et al., 2013).

THP-1 cells remain a valuable and informative biological system for the study of monocytes and macrophages, however, key differences in their inherent cancerous biology and the consequence of constant *in vitro* culture make the relationships between primary monocytes and THP-1 cells one of resemblance rather than a system that perfectly mimics the physiological reality. Notable differences between THP-1 cells and primary monocytes have been reported in studies that measured basal gene expression and responses to stimulation/activation (Kohro et al., 2004). Of particular interest within the context of this

study is the effect of type I/II interferon on the basal expression of key genes within THP-1 cells and CD14+ healthy patient monocytes.

As discussed in detail above, the interferon response protects against microbial invasion and has been shown to both directly inhibit the growth of viruses and bacteria as well as play a significant role in the priming of the immune system for a protective response. Almost 60 years ago, interferon was first discovered as a secreted factor from virally infected cells which elicited a protective response in the immediate cellular environment by preventing subsequent infection (Isaacs and Lindenmann, 1957). The large group of secreted factors was termed interferons due to their ubiquitous ability to stimulate the transcription and upregulation of antiviral and antibacterial gene motifs which in turn facilitated host protection (Decker et al., 2005). In mouse knockout studies, animals with an IFN deficient phenotype were highly susceptible to microbial challenge and displayed increased mortality (Schroder et al., 2004, Huang et al., 1993). The classification of two interferon types, type I and II, is based on the cell surface receptors to which they preferentially bind. Type I IFN bind to the IFN alpha receptor (IFNAR), which consists of two subunits; IFNAR1 and IFNAR2. Type II IFN binds to the IFNγ receptor (IFNGR) which is composed of two chains; IFNGR1 and IFNGR2 (Sivro et al., 2014). Both receptors modulate antimicrobial gene expression via the JAK-STAT signalling pathway (Decker et al., 2005). Both types also regulate the differentiation of T-helper cells impacting T-cell immunity in addition to the innate immune responses (Decker et al., 2005).

Type I IFNs, of which there are 20, are predominantly involved in the antiviral response during viral infection of the host (Decker et al., 2005). In mammals, IFN-α, IFN-β, IFN-ϖ, IFN-κ and IFN-ɛ are the predominant type I IFN genes (Decker et al., 2005). Conversely, the type II IFN family consists of only one member IFNγ, which is primarily associated with antibacterial immunity. IFN-γ is primarily secreted by IL-12 induced Th1 T-cells. Individuals with deleterious mutations in the IL-12 receptor as well as mouse knockout studies of IFN-γ have shown that IFN-γ and IL-12 deficient subjects were more susceptible to *M. tb* infection, with more severe morbidity and increased mortality (Cooper et al., 1997, Cooper et al., 1993).

Recent studies of the entire transcriptome of peripheral blood elucidated gene expression profiles in which type I/II IFN signalling pathways correlate with the latent and active forms of tuberculosis (Ottenhoff et al., 2012). The idea that type I/II signalling plays a role in mycobacterial pathogenesis is further supported by evidence from leprosy where Type II interferon has been shown to facilitate the upregulation of CAMP and DEFB4 mRNA via the vitamin D-dependent antimicrobial pathway as well as encouraged significant antimicrobial activity in human monocytes. These authors found that this protective effect was abrogated by the type I interferon which reduced mRNA levels of both antimicrobial peptides and antimicrobial activity (Teles et al., 2013).

These observations suggest that interferon signalling could have a significant impact on mycobacterial immunity in the context of HIV-infected individuals. In both the acute and chronic phases of HIV numerous immunoregulatory cytokines, including the interferons, are deranged (Stacey et al., 2009). Of notable importance, particularly in light of the work done in leprosy, is type I interferon, which remains at elevated levels through to the chronic phase of infection (Stacey et al., 2009, Hardy et al., 2013).

The fact that TB risk increases in HIV individuals prior to a significant depletion of the CD4 T-cell population implies that the loss of immune control of *M.tb* is at least partially independent of CD4 loss (Sonnenberg et al., 2005). Thus, the imbalance of type I/II interferon observed in the co-infected cellular milieu may be a contributing factor to the loss of host control and progression to TB disease. However, the exact mechanism by which HIV-induced type I interferon may alter host IFN-γ mediated TB control in HIV-1 positive individuals remains an important gap in knowledge in the field.

We initially hypothesised that the basal expression of the transcripts of interest would be comparable in primary monocytes from healthy donors and THP-1 cells, making THP-1 cells a useful model for the study of this pathway and its modulators. Further, knowing that HIV infection induces an immune milieu that is skewed towards Type-I IFN, we hypothesised that this would result in decreased production of the Vitamin D-dependent antimicrobial peptides. As such, we hypothesised that exogenous application of Type-I IFN (at a physiologically relevant concentration similar to that seen in HIV infection)

to innate phagocytes *ex vivo* was expected to downregulate key antimicrobial peptides when compared to unstimulated or Type-II stimulated cells.

Within the context of the Vitamin-D dependent antimicrobial protective pathway, the aim of this study was to assess the basal expression of CAMP, VDR, CYP24a1, CYP27b1 and DEFB4 relative to the reference gene GAPDH in the THP-1 cell line and primary CD14+ monocytes and determine how this expression was modulated by type I/II interferon. This was achieved utilising the optimised real-time PCR assays detailed in Chapter Two of this thesis.

3.1 Methods and Materials

3.1.1 Cells and Reagents

3.1.1.1 THP-1 Cells

THP-1 cells were stored, thawed and maintained as described in 2.2.1.1. Approximately 2 million cells were isolated and stimulated per condition (rhIFNγ (2000 IU/mL) and rhIFNα2a (2500 IU/mL) respectively) or left unstimulated for 24 hours at 37° C. Concentrations and duration of stimulation using interferon were based on the literature and previously experiments in our laboratory; the concentrations were chosen to reflect the physiological concentrations observed during peak viral and bacterial infection (Gomez et al., 2015, Herbst et al., 2011).

3.1.1.1 PBMC Isolation

Healthy-donor leukopaks were acquired from donors from the South African National Blood Service in Durban, South Africa. These donors were free from all known pathogens for which the South African National Blood Service screens. Buffy coats were layered onto an equal volume of Histopaque® (Sigma-Aldrich) and spun at 400 x g for 45 minutes. Human plasma was removed to a separate tube. The PBMC layer was removed to 45 mL Dulbecco's phosphate-buffered saline (DPBS) and spun at 300 x g for 10 minutes. The supernatant was discarded and another washing step was done. Sample supernatant was

discarded, the cells were resuspended in 10 mL R10 and manually counted. PBMCs were then rested for two hours prior to CD14 positive selection of monocytes.

3.1.1.2 CD14+ Cells

Monocytes from the PBMC preparation were isolated via positive immunomagnetic selection using CD14 MicroBeads kit (Miltenyi Biotec, Germany). Briefly, PBMCs were counted before being spun down at 300xg for 10 minutes. The supernatant was completely removed via aspiration. The pellet was resuspended in MACS buffer (2mM ethylenediaminetetraacetic acid (EDTA), 1% HI-FBS in PBS) to 80 μL per 10^7 total cells. 20 μL of CD14 MicroBeads was added per 10^7 cells. The sample was then mixed well and incubated at 2-8 °C for 15 min. After incubation, the cells were washed by adding 2 mL MACS buffer per 10^7 cells and spun at 300 x g for 10 minutes, after which the supernatant was completely aspirated. Cells were resuspended in 500 μL MACS buffer per 10^8 cells. MACS LS Separator Column was placed in the magnetic field and rinsed with 3 mL MACS buffer. The sample was placed in a MACS LS Separator Column and the cell suspension was allowed to pass through the column and the column washed three times with 3 mL MACS buffer. The column was then removed to separate collection tube and 5 mL MACS buffer was used to flush out CD14+ cells.

Using the same protocol for THP-1 cells and primary monocytes, 2 million CD14+ cells were stimulated per condition (rhIFNγ (2000 IU/mL) and rhIFNα2a (2500 IU/mL) respectively) or left unstimulated for 24 hours at 37° C. Concentrations and duration of stimulation using interferon were based on previously conducted studies and are assumed to reflect the physiological concentrations observed during peak viral and bacterial infection (Gomez et al., 2015, Herbst et al., 2011).

3.1.2 RNA Isolation and cDNA Synthesis

Cells were lysed, RNA isolated and reverse transcription were performed as described in 2.2.2.2.

3.1.3 Primers and Real Time PCR

Primer selection and real-time PCR conditions were described in section 2.2.3. Biological replicates were performed in triplicate (for THP-1 cells) and three independent healthy donors were assessed (for primary monocytes). Technical replicates were performed in duplicate for each condition and each target gene.

3.1.4 Analysis of Gene Expression

The basal expression of transcripts of interest was expressed relative to GAPDH using the relative standard curve method (Larionov et al., 2005). The impact of interferon stimulation on gene expression in THP-1 cells and CD14+ healthy patient monocytes was calculated relative to unstimulated controls using the Pfaffl equation and normalised to GAPDH (Pfaffl, 2001).

The significance of relative gene expression between stimulation conditions was analysed using an ANOVA test in GraphPad Prism (version 6). Assumptions for ANOVA, normality and equal variance of residuals, were verified by a one-sample Kolmogorov-Smirnov test and Levene's test respectively. Statistical tests were performed using 0.05 level of significance. The Tukey test was used to correct for multiple comparisons.

3.2 Results

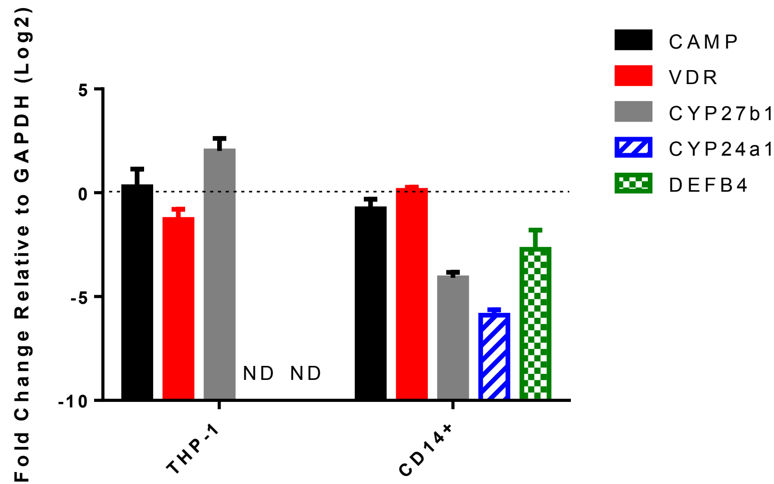


Figure 30: Basal Expression of Transcripts of Interest Relative to GAPDH is Variable in THP-1 Cells and CD14+ Healthy Patient Cells.

3.2.1 Comparison of basal expression level for transcripts of interest in THP-1 cells and primary monocytes

As shown in Figure 30, three genes (CAMP, VDR and CYP27b1) were detectable in both THP-1 cells and unstimulated primary CD14+ monocytes. CAMP and VDR were expressed at similar levels (relative to GAPDH) in THP-1 cells and monocytes. Notably, CYP27b1 was expressed at a notably lower level (relative to GAPDH) in primary monocytes than in THP-1 cells. Also of note, CYP24a1 and DEFB continued to be undetectable in THP-1 cells (as discussed in section 2.2.6) but were detectable in primary CD14+ monocytes.

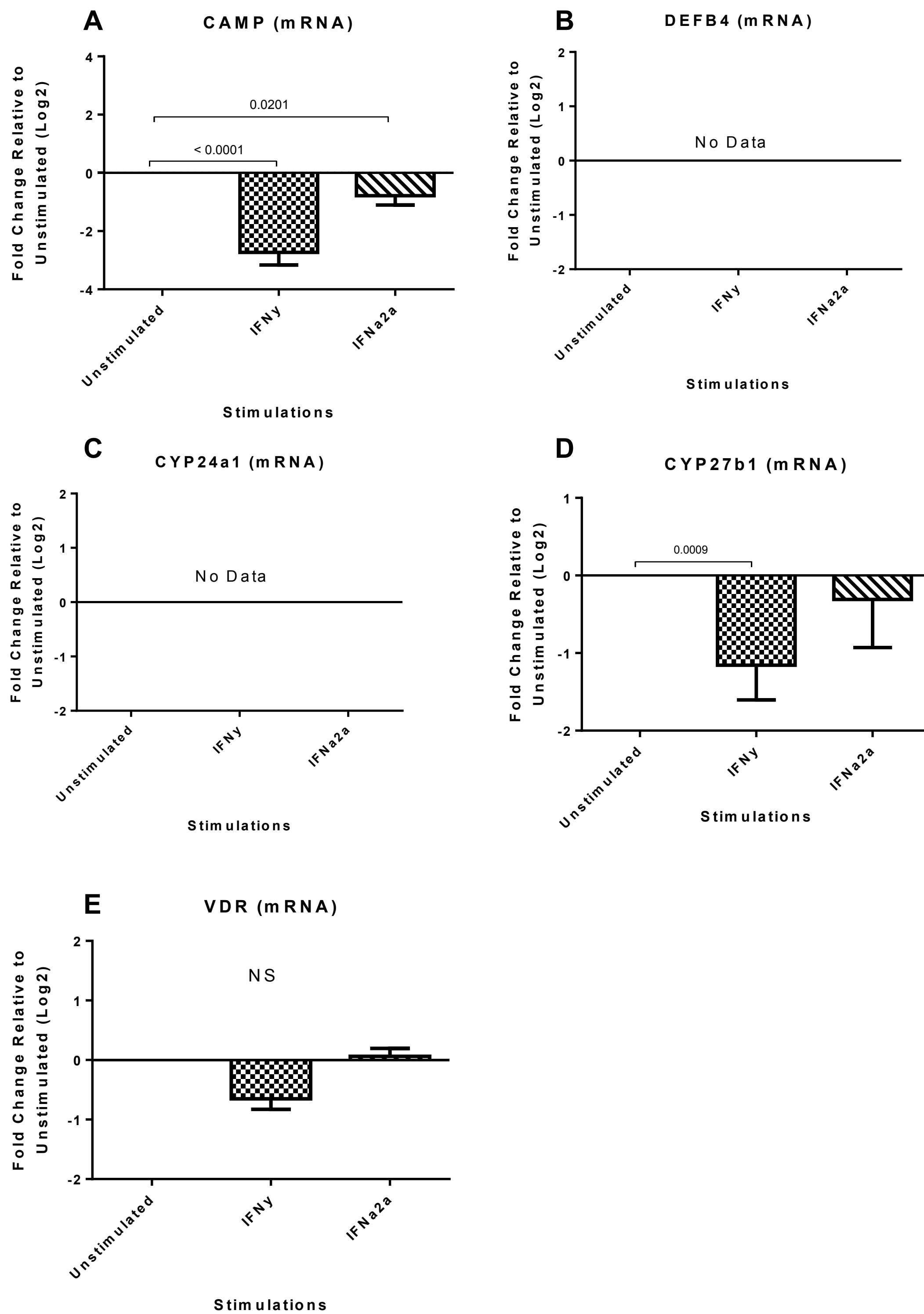


Figure 31: Modulation of Gene Expression via Type I (2500 IU/mL) /II (2000 IU/mL) Interferon Stimulation of THP-1 Cells over 24 hours (n=3).

3.2.2 Impact of interferon stimulations on gene expression in THP-1 cells

As shown in Figure 31, THP-1 cells were stimulated with (rhIFNγ (2000 IU/mL) and rhIFNα2a (2500 IU/mL) for 24 hours. Relative to unstimulated controls, cathelicidin (CAMP) was significantly downregulated by both type I interferon ($p=0.0201$) and type II interferon ($p<0.0001$). CYP27b1 expression was significantly downregulated by type II interferon ($p=0.0009$) but not altered by type I interferon. Vitamin D Receptor (VDR) was not significantly modulated by either stimulation ($p>0.05$). As discussed previously, beta-defensin 2 (DEFB4) and CYP24a1 were undetectable in THP-1 cells.

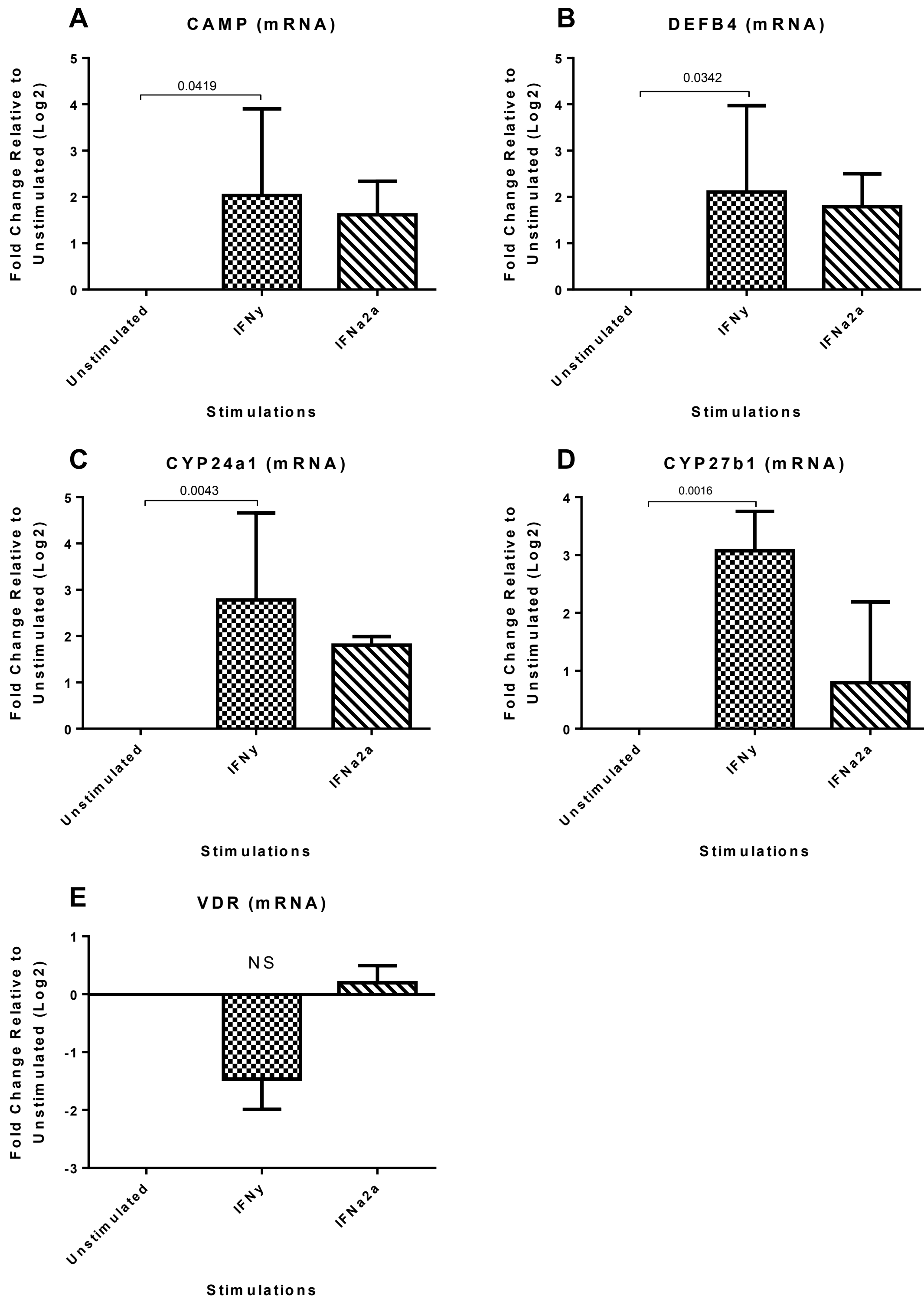


Figure 32: Modulation of Gene Expression via Type I (2500 IU/mL) /II (2000 IU/mL) Interferon Stimulation of Healthy Patient CD14+ Monocytes (n=3).

3.2.3 Impact of interferon stimulations on gene expression in primary CD14+ monocytes

As shown in Figure 32, healthy donor CD14+ monocytes were stimulated with (rhIFNγ (2000 IU/mL) and rhIFNα2a (2500 IU/mL) for a period of 24 hours. Although significant effects due to interferon stimulation were detected, it is worth acknowledging that interpatient variability may be the major driver of differences between stimulations observed in our primary cell data. Despite this, cathelicidin (CAMP) was significantly up-regulated by type II interferon ($p=0.0419$) and not modulated by type I interferon. Beta-Defensin 2 (DEFB4) was significantly up-regulated by type II interferon ($p=0.0342$) and not modulated by type I interferon. CYP24a1 was significantly up-regulated by type II interferon ($p=0.0043$) and not modulated by type I interferon. CYP27b1 was significantly up-regulated by type II interferon ($p=0.0016$) and not modulated by type I interferon. Vitamin D Receptor (VDR) was not significantly modulated by either type I or type II interferon stimulation.

3.3 Conclusions and Discussion

The changing interferon environment observed in bacterial and viral coinfections may have a significant impact on the pathogenesis of the disease. Mouse and human studies which have examined the effect of type I interferon, IFN-β, on mycobacterial infection have observed increased bacterial loads as well as increased mortality (McNab et al., 2014, Teles et al., 2013, Bouchonnet et al., 2002). In the context of tuberculosis, protective host-mediated immune control of *M. tb* microbes can be partially attributed to a vitamin D-dependant antimicrobial pathway, which is under the regulation of type II interferon (IFN-γ) in monocytes and macrophages in healthy individuals (Modlin and Bloom, 2013, Rook et al., 1986, Sonawane et al., 2011, Teles et al., 2013). Previous studies have linked an increase in type I interferon with a decrease in the transcription of key antimicrobial peptides and a worsening of bacterial control (Shahangian et al., 2009, Teles et al., 2013)

During acute HIV-1 infection, there is a global upregulation of multiple immunoregulatory cytokines in a phenomenon termed cytokine storm (Stacey et al., 2009). Of particular interest within the

scope of this study is the initial, and chronic, upregulation of the type I interferon, IFNα2a, which is not only measurable at elevated levels in the blood but is actively expressed by alveolar macrophages in the lung (Hardy et al., 2013, Kumagai et al., 2007). Previous studies on type I/II interferon imbalance have primarily focused on the effect of IFN-β, the key type I interferon upregulated during other notable viral infection such as influenza A (Shahangian et al., 2009, Teles et al., 2013). As such, the effect of IFNα2a within the context of protective immune control of mycobacterial/HIV coinfection remains a key gap in knowledge of this field since it is the key type I interferon was shown to be elevated in HIV-infected patients (Hardy et al., 2013).

In the current study, we measured the independent effects of recombinant IFNα2a and IFN-γ on the transcription of antimicrobial peptides (CAMP and DEFB4) and associated regulatory transcripts (VDR, CYP24a1 and CYP27b1) in the human monocytic cell line THP-1 and CD14+ monocytes. To our knowledge, a study looking at the effect of type I/II interferon imbalance, specifically in the context of HIV-1 induced IFNα2a has not been conducted in a South African cohort. Although limited by the small sample size, this study has provided interesting insights into the potential effects an imbalanced type I/II interferon immune milieu expected in HIV/TB coinfection.

3.3.1 Expression of the pathway of interest differed in THP-1 cells and primary CD14+ monocytes

The basal expression of a transcript of interest in a healthy cell can be used as a measure of its developmental state or its ability to quickly translate certain transcripts upon activation. In the current study, we have shown that healthy, uninfected CD14+ human monocytes and THP-1 cells, an immortalised monocytic cell line, differ in basal expression of key antimicrobial peptides and regulatory transcripts. Only two of five transcripts of interest demonstrated similar levels of baseline transcription (relative to the housekeeping gene) in THP-1 cells and CD14+ human monocytes. CAMP and VDR were comparably expressed relative to GAPDH in both THP-1 cells and primary human monocytes. One of the five transcripts of interest, CYP27b1, was expressed at a notably lower level in CD14+ monocytes than in THP-

1 cells. Interestingly, in THP-1 cells the expression of CYP24a1 and DEFB4 was reproducibly undetectable. However, in primary CD14+ monocytes, CPY24a1 and DEFB4 were detectable, though at low levels compared to GAPDH and the other transcripts of interest.

Our finding, that CYP24a1 and DEFB4 were undetectable in THP-1's has been noted in the literature (Liu et al., 2007, Adams et al., 2009, Gottfried et al., 2006). Specifically, Adams *et al* (2009) and Gottfried *et al* (2006) were unable to detect DEFB4 and CYP24a1 in cells cultured in Vitamin D-deficient media. However, when vitamin D was supplemented (100 nM) these authors were able to detect these transcripts. Based on this, we think it likely that our results are due to culture in a vitamin D deficient environment.

Under the experimental conditions that we used (no vitamin D supplementation), it is clear that THP-1's were not a very good model for the study of this pathway in primary CD14+ monocytes. This highlights the importance of testing any model system prior to assuming that it is reflective of *ex vivo* primary cells. Based on these results, it might seem advantageous to solely study this pathway in primary cells, however, intra-patient variation would confound results and limit reproducibility. Although the work presented here did not solve this perennial problem, it does highlight the importance of interrogating cell lines for the measure of interest against the primary cells to understand the limitations of any given model system.

3.3.2 Interferon gamma upregulated key immune factors in CD14+ monocytes and unexpectedly down-regulated some of the same factors in THP-1 cells

Our results from healthy CD14+ monocytes showed significant upregulation of CAMP, DEFB4 and CYP27b1 upon type II IFN (rhIFN-γ) stimulation; this is in agreement with previously published studies (Fabri et al., 2011, Teles et al., 2013). The upregulation of these transcripts implies the expected functioning of the vitamin D-dependent antimicrobial pathway within healthy patient CD14+ cells (see Figure 32 below).

In contrast, CAMP and CYP27b1 were downregulated by type II IFN stimulation in THP-1 cells. This was unexpected, but, as discussed above, our results showed notable differences between baseline levels of transcription of these genes in the cell line and primary monocytes. It is possible that the downregulation of these two genes in THP-1 cells reflects vitamin D deficient conditions. Previous studies have shown the direct link between this vitamin and CAMP induction (see Figure 32 below) and linked vitamin D deficient conditions to dysfunction of this pathway (Gombart et al., 2005, Schauber et al., 2008, Gonzalez-Curiel et al., 2014). Along similar lines, our inability to detect DEFB4 and CYP24a1 in THP-1 cells may also be attributed to vitamin D deficient conditions in THP-1 cells (Fabri et al., 2011). The potential effect of vitamin D availability will be further discussed in section 3.3.4 of this discussion.

3.3.3 Interferon Alpha 2a stimulation did not significantly modulate the expression of key immune factors in CD14+ cells

Exogenous Type I interferon (rhIFNα2a) stimulation of CD14+ cells revealed unexpected directionality of gene modulation. In healthy donor CD14+ cells, all genes with the exception of VDR showed a non-significant trend towards up-regulation after Type I IFN stimulation (see Figure 32 below).

Exogenous Type I interferon (rhIFNα2a) stimulation of THP-1 cells resulted in downregulation of the pathway of interest. In THP-1 cells, exogenous rhIFNα2a stimulation resulted in the significant downregulation of CAMP and the non-significant trend towards downregulation of VDR and CYP27b1 (see Figure 32 below). This result is similar to those found in studies that utilised IFNβ as the primary source of type I interferon (Teles et al., 2013).

Though nonsignificant, the upregulation of the pathway in CD14+ monocytes is in contrast to the published literature which reports downregulation of this pathway upon *in vitro* stimulation with Type I IFN. A possible explanation for this discrepancy could be differences in effect between IFNα2a and IFN-β. Although both interferons bind the same cellular receptor, IFNAR1, they may have varying effects on the vitamin D-dependent antimicrobial peptides and regulatory transcripts. Although it was beyond the scope of this project, the next step would be to directly test the hypothesis that IFNα2a and IFN-β have

different effects on this pathway in primary CD14+ cells. If confirmed, this line of investigation could have implications for the antimicrobial potential of these cells in the context of an overexpression of IFNα2a in HIV-1 infection.

That our findings contrast to those previously reported could also have a genetic explanation. Recent studies have highlighted the effect of genomic single nucleotide polymorphisms which vary between populations of European and African origin and alter the expression of genes (up to 9.3% in macrophages) involved with immune response and inflammation when challenged with microbial stimulants/agonists (Quach et al., 2016, Nedelec et al., 2016). In general, the majority of studies which have looked at the effect of type I/II interferon imbalance on this pathway have been performed in settings in which the healthy donors were likely of European ancestry (Adams et al., 2009, Fabri et al., 2011, Teles et al., 2013). Thus, healthy donors of African ancestry may elicit a different response to interferons and this area of research represents a significant and interesting gap in scientific knowledge and may to some extent explain the variability in our results compared to the literature. Small sample size represents another important potential explanation for the differences observed in our study compared to the literature. It is possible that with more independent donors, a significant modulation by IFNα2a would have been observed. To overcome both of these issues the next step would be to directly compare the gene modulation between and an appropriate number of donors of both African and European ancestry.

The non-significant modulation (towards upregulation) of CYP24a1 by IFNα2a in healthy donor CD14+ cells was an interesting observation particularly in the light of leprosy studies which found that upregulation of CYP24a1 was correlated with the expression of IL-4 at the site of lesions (Yamamura et al., 1991, Cooper et al., 1989). Within our South African healthy donor CD14+ samples, CYP24a1 was significantly upregulated via stimulation with IFN-γ - independent of CD4+ T cells implying a direct inductive effect via IFN-γ in the absence of IL-4. This interaction may be facilitated via a direct gene-to-gene effect, however, it likely involves several other contributing factors and genes. Again, although this hypothesis is beyond the scope of this project, the next step would be a direct comparison of the effect of

rhIFN-γ and exogenous IL-4 on CYP24a1 expression within primary monocytes. If confirmed, this may elucidate alternative regulatory pathways for vitamin D metabolism under the control of type II interferon and would have a direct effect on the protective vitamin D-antimicrobial response. More comprehensive hypothesis generating transcriptomic analyses, such as RNA-seq, may be useful avenues of experimentation to tease out unknown contributors to protection, regulation or sensitivity.

The detection of CYP24a1 at the basal level in healthy donor CD14+ cells may be indicative of some form of catabolic vitamin D negative feedback mechanism of this protective pathway due to excess 25 vitamins D3- as these healthy donors are assumed to be vitamin D replete.

In summary, stimulation of healthy donor CD14+ cells with rhIFN-γ resulted in the upregulation of protective AMPs and key regulatory factors in support of our original hypothesis. Stimulation with rhIFNα2a, though non-significant, induced pathway genes in an unanticipated manner. In THP-1 cells, and in contrast to literature as well as our original hypothesis, rhIFN-γ unexpectedly downregulated key pathway genes and regulatory transcripts while rhIFNα2a had the anticipated negative modulation of the pathway. The expected results observed in this study may be as a consequence of vitamin D deficiency, the differing activity of IFNα2a vs. IFNβ, absent TLR 1/2 stimulation or population genetics.

3.3.4 The role of Vitamin D may explain differing basal expression as well as unexpected programs of gene modulation between CD14+ cells and THP-1 cells

One of the key findings of this study was the difference in transcription of key genes in the pathway of interest observed between THP-1 cells and primary monocytes. CYP24a1, as well as DEFB4, could not be detected in THP-1 cells. Culture in a vitamin D deficient cellular environment may have contributed to the lack of expression of DEFB4, which has been hypothesised to be induced by the binding of the bioactive form, 1, 25 vitamin D to the vitamin D receptor (VDR). Interestingly, HIV-infected persons worldwide have been found to suffer from chronic vitamin D deficiency which may hamper the expression of key antimicrobial peptides resulting in increased sensitivity to TB disease and increased mortality even when

cART is initiated (Mueller et al., 2010, Allavena et al., 2012). Fabri *et al.* found in their 2011 study that exposure to exogenous 1, 25D3 in conjunction with rhIFNγ stimulation significantly increased the expression of both CAMP and DEFB4 in human monocytes and macrophages by approximately 6 and 5.5 fold respectively (Fabri et al., 2011). However, under vitamin D deficient conditions IFN-γ stimulation either significantly downregulated CAMP and DEFB4 or showed no significant difference to negative controls, a phenomenon observed in the THP-1 results of this study (Fabri et al., 2011). Similarly, in THP-1 cells, Liu *et al.* found that expression of CYP24a1 was detectable only under culture conditions containing 1, 25D3, a phenomenon which has been observed in several studies (Liu et al., 2007, Heulens et al., 2016).

Vitamin D metabolism plays an important role in our pathway of interest and varying levels of the bioactive forms of vitamin D in different samples may help to explain our findings. The low levels of basal CYP27b1 and DEFB4 quantified in the CD14+ monocytes were unsurprising when taken in the context of a "healthy individual" who is assumed to be vitamin D replete. In the absence of inflammatory disease and the presence of sufficient vitamin D, the upregulation of vitamin D metabolism and antimicrobial pathways would not necessarily be required and would exist at a normal basal level. This interpretation of the data is further supported by the slight upregulation of VDR that we observed in CD14+ cells compared to THP-1 cells; VDR upregulation is observed during physiological excess of bioactive vitamin D (Adams et al., 2009).

In the experiments presented here, THP-1 cells were not supplemented with any form of Vitamin D. It is possible that in THP-1 cells, the upregulation of CYP27b1 and the undetectable expression of CYP24a1 and DEFB4 could be attributed the long-term culture of THP-1 cells in a Vitamin-D deficient environment. Adam *et al.* 2009 have reported similar data in human monocytes cultured *in vitro* under Vitamin-D deficient conditions resulting in the 5-fold upregulation of CYP27b1. They hypothesised that a lack of the circulating form of Vitamin-D, 25-hydroxyvitamin D ($25(OH)D_3$), prompted the upregulation of this gene in an attempt "prime" the cell for potential antimicrobial activity via the rapid scavenging of environmental Vitamin-D.

The detectable basal expression of CAMP within THP-1 cells was an interesting finding in our experiments, as upregulation of CAMP and DEFB4 has been linked to the binding of 1, 25 vitamin D3 to the vitamin D receptor (VDR) (Adams et al., 2009, Fabri et al., 2011, Gombart et al., 2005, Heulens et al., 2016). If the hypothesis of DEFB4 downregulation/loss in the face of vitamin D deficiency is correct, then the expression of CAMP in the absence of vitamin D implies a possible alternative pathway in THP-1 cells that maintain expression of CAMP even in Vitamin-D deficient conditions.

The presence or absence of vitamin D may modulate the host immune response. In data shown in this study, it is likely that the unexpected results observed between THP-1 cells and primary monocytes may be as a consequence of vitamin D deficiency. The observed absence of induction of protective genes in THP-1 cells, that are cultured in a vitamin D lacking environment, is particularly interesting when taken in the context of HIV-1 infected individuals who are generally chronically vitamin D deficient and may explain sensitivity to TB disease. These observations raise interesting questions about antimicrobial peptide expression in the context of varying Vitamin D levels but go beyond the scope of this Master's project. However, to better elucidate the effect of vitamin D on this pathway the next steps would be the assessment of this protective pathway via the measurement and standardisation of vitamin D conditions in future *in vitro* and *ex vivo* studies.

3.3.5 Overall Conclusions

In conclusion, we assessed the basal expression of CAMP, VDR, CYP24a1, CYP27b1 and DEFB4 in the THP-1 cell line and healthy donor CD14+ cells and found notable differences between expression levels of these transcripts in the two sets of cells (Figure 33). CYP24a1 and DEFB4 were undetectable in the THP-1 cell line but detectable in primary monocytes and CYP27b1 was expressed at significantly higher levels relative to housekeeping genes in THP-1 cells compared to CD14+ cells. We hypothesise that these findings may be a consequence of the culture of the THP-1 cells in a Vitamin-D-deficient cellular environment. We conclude that under the tested conditions, THP-1 cells have significant limitations as a model system for studying the immune mechanisms of control of *M. tb* in monocytes.

In terms of the effect of exogenous interferon stimulation, we have shown, despite the small sample size, several interesting outcomes relating to the effect of type I/II interferons on human monocytes. Stimulation of CD14+ cells *ex vivo* with rhIFNγ resulted in the up-regulation of key antimicrobial peptides and regulatory transcripts important in inducing a protective innate response against pathogenic microbes, while type I interferon (rhIFNα2a) had no significant effect on the expression of the same peptides in primary monocytes and THP-1 cells but showed an unanticipated trend towards upregulation of the antimicrobial peptides. The unexpected downregulation of transcripts of interest in THP-1 cells upon stimulation with rhIFNγ is hypothesised to be as a result of vitamin D deficiency, thus highlighting the importance of this vitamin in the context of this protective pathway.

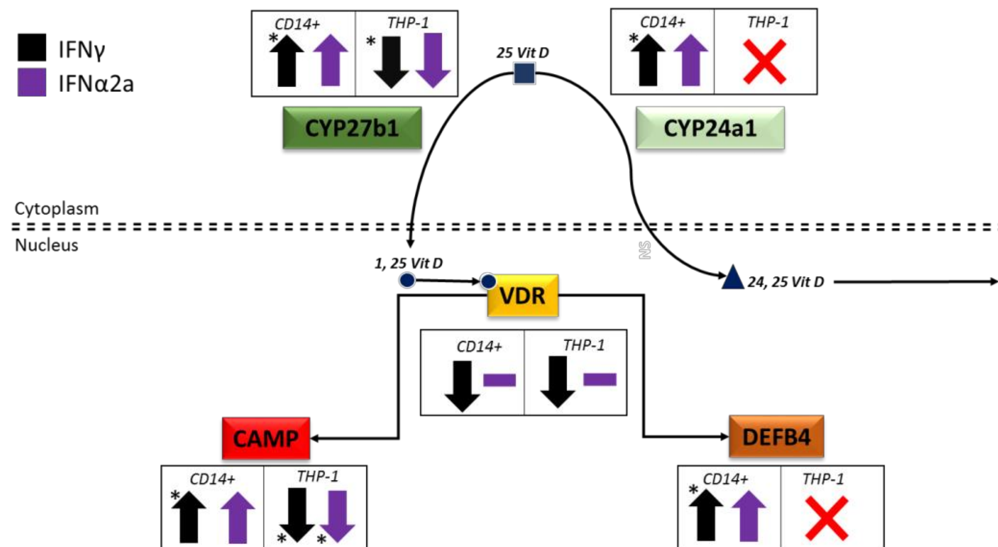


Figure 33: Summarised Effect of Type I/II Interferon on the Expression of Key Antimicrobial Peptides and Regulatory Transcripts of the Vitamin D-Dependent Antimicrobial Pathway in THP-1 cells and Healthy Patient CD14+ Monocytes. Black arrows/bars equate to modulation or no effect of Type II IFN (IFNγ) respectively. Purple arrows equate to modulation of Type I IFN (IFNα2a) respectively. *$p<0.05$

This study was designed to examine the vitamin D-dependent antimicrobial pathway and the independent impact of type I/II interferon in monocytes. Results from this study have highlighted the potentially critical effect of uncontrolled for co-factors on the modulation of key genes and regulatory

transcripts. Specifically, as discussed above, Vitamin D is almost certainly of central importance in this pathway. Additionally, it is likely that other known modulators of the pathway -- like the TLRs -- are important. In the complicated context of HIV infection and a disturbed inflammatory milieu, there may be additional other modulators of this pathway. Nonetheless, this study confirms IFN-γ as an inducer of this pathway and raises many questions about the potential role of Type 1 IFN on this pathway and in the context of HIV infection. Although much needs to be worked out, it seems clear that Vitamin D deficiency and HIV may together have a deleterious impact on this antimicrobial pathway in human monocytes. The groundwork for further study of this important topic has been laid through the methodological work presented here.

4 CHAPTER FOUR: General Discussion and Conclusions

South Africa has one of the world's highest incidents of both HIV and TB, with TB being the leading infectious killer of HIV-infected persons both within this country and worldwide (WHO, 2014, WHO, 2016a). Globally it is estimated that approximately one in three people is infected with *M. tb*, however, only a small portion (approximately 10%) of those who are infected will ever progress to active disease in their lifetimes (Rappuoli and Aderem, 2011, WHO, 2016a). Immunosuppressive conditions such as diabetes, TNF neutralisation therapies and, in particular, HIV infection, have all been linked to reactivation of TB disease in those who have suffered in the past as well as the novel pathogenesis of disease in immunosuppressed individuals (Barry et al., 2009).

The large number of otherwise healthy latently infected individuals relative to those who progress to active TB disease, particularly in high TB burden countries such a South Africa, implies the presence of host-mediated protective mechanisms capable of inhibiting pathogenesis. This host driven control can at least be partially attributed to the Vitamin-D dependent antimicrobial pathway in monocytes and other phagocytes (Modlin and Bloom, 2013, Campbell and Spector, 2012, Chun et al., 2015, Liu et al., 2007). This protective pathway results in the downstream induction of the antimicrobial peptides CAMP and DEFB4 via a number of intermediate regulatory genes.

Regulation of this pathway is mediated by the presence of the type II interferon, interferon gamma which is primarily secreted by T helper cells during bacterial infection. Effective immune control of *M. tb*, therefore, requires a robust T-helper type 1 (T_h1) immune response, which has both a direct anti-TB effect but also dictates the accompanying innate immune responses of macrophages and other antigen presenting cells. The importance of IFN-γ has been convincingly illustrated in mouse knockout models which have shown a significant increase in the sensitivity to *M. tb* infection as well as increased mortality.

Conversely, it has been shown that type I interferon, which is secreted in response to viral infection, has the ability to abrogate the protective response of the Vitamin-D dependent antimicrobial pathway by downregulating key antimicrobial peptides facilitating a loss of immune control (Teles et al., 2013). This is true for most temporal viral infections such as influenza, however, chronic infection such as HIV have implications for TB pathogenesis (Redford et al., 2014). HIV-1 has been shown to be a potent inducer of numerous immunoregulatory cytokines which never return to baseline expression throughout the course of the disease (Stacey et al., 2009). Of particular importance for the Vitamin-D pathway is the chronic upregulation of the type I interferon, IFNα2a (Hardy et al., 2013). The quantification of the degree to which the antimicrobial peptides and associated factors are modulated by an unbalanced interferon milieu observed in TB/HIV coinfection was the ultimate goal of this study.

The assessment of the vitamin D-dependent antimicrobial pathway requires a robust molecular toolkit for reliable quantification of key genes. In this study, a one plate real-time PCR assay for the accurate and reproducible detection of CAMP, VDR, CYP24a1, CYP27b1 and DEFB4 using SYBR green chemistry was optimised for use in THP-1 and primary cells. Primer pairs were bioinformatically validated and amplicons were run on a 2.5% agarose gel to ensure correct product sizes. Interesting, CYP24a1 and DEFB4 were reproducibly undetectable in THP-1 cells and the primer pairs for each gene had to be optimised using a commercial reference RNA library made of 10 different human tissues which were later validated in healthy patient primary monocytes. The lack of expression of both genes is thought to be as a result of the

long-term culture of the THP-1 cells in a Vitamin-D deprived environment, though this hypothesis still remains to be confirmed.

The optimisation of a one plate real-time PCR assay for key genes in this protective pathway facilitated the assessment of both the basal expression levels of these genes as well as the independent effect of exogenous recombinant type I/II interferon stimulation on both THP-1 cells and healthy patient CD14+ monocytes. Basal expression levels revealed that CAMP, VDR and CYP27b1 were detectable in both THP-1 cells and primary CD14+ monocytes. CAMP and VDR were expressed at similar levels (relative to GAPDH) in THP-1 cells and monocytes. Notably, CYP27b1 was expressed at a significantly lower level in primary monocytes than in THP-1 cells. This may be explained by the vitamin D deficient culture environment where the cells are actively trying to ramp up their vitamin D metabolism. This result is in agreement with a study conducted by Adam *et al.* 2009 who noted a 5.5-fold increase in CYP27b1 in human monocytes cultured under vitamin D-deficient conditions. Also of note, CYP24a and DEFB continued to be undetectable in THP-1 cells but were detectable in primary CD14+ monocytes.

The independent stimulation of THP-1 and CD14+ healthy patient monocytes with type I/II interferon displayed differing programs of gene modulation. In THP-1 cells, IFNα2a induced no significant effect on the three detectable transcripts of interest (CAMP, CYP27b1 and VDR) whilst stimulation with IFN-γ resulted in the unexpected down-regulation of CAMP and CYP27b1. In healthy donor CD14+ monocytes stimulated with IFN-γ, CAMP, CYP24a1, CYP27b1 and DEFB4 were all significantly upregulated. IFNα2a resulted in a trend towards up-regulation of CAMP, CYP24a1, CYP27b1 and DEFB4. VDR expression was not modulated by either Type I or II interferon stimulation in healthy donor monocytes.

In conclusion, I have developed a one plate real-time PCR assay for the accurate quantification of CAMP, VDR, CYP24a1, CYP27b1 and DEFB4 – key genes in the vitamin D-antimicrobial pathway. Utilising this optimised assay, and despite a small sample size (n=3), I discovered notable differences in the basal expression of these genes in the monocytic cell line; THP-1 cells and primary CD14+ monocytes.

Further, exogenous recombinant type I/II interferon stimulation of THP-1 cells and patient monocytes also displayed varying programs of gene modulation. Primarily, *ex vivo* stimulation of healthy patient primary monocytes with type II interferon (rhIFNγ) resulted in the up-regulation of key antimicrobial peptides and regulatory transcripts important in inducing a protective innate response against pathogenic microbes. Unexpectedly, type I interferon (rhIFNα2a) had no significant effect on the expression of the same peptides in primary monocytes and THP-1 cells but showed an unanticipated trend towards upregulation of the antimicrobial peptides, CAMP and DEFB4. The unexpected downregulation of transcripts of interest in THP-1 cells upon stimulation with rhIFNγ is hypothesised to be as a result of vitamin D deficiency, thus highlighting the importance of this vitamin in the context of this protective pathway, particularly in light patient samples, however, this hypothesis remains to be confirmed. Further, we have demonstrated an impaired ability of THP-1 cells to amplify DEFB4 *in vitro* implying a key disadvantage for the use of this cell line as a model for relevant antimicrobial activity studies as well highlighting key differences between cell lines and primary cells.

5 CHAPTER FIVE: Limitations and Future Studies

The specific limitations observed in this study, and potential solutions to overcome these limitations in the future, have been mentioned in the discussion sections of Chapters 2 and 3 above. However, in short, additional experiments, using an appropriately large data set, and next steps should focus on;

- Evaluating the effect of bioactive vitamin-D on this antimicrobial pathway by; a) quantifying its concentration in plasma and b) conducting stimulation experiments with/without exogenous bioactive vitamin-D.
- Evaluating the effect of activation through the stimulation of TLR 1/2 using appropriate ligands in conjunction/absence of Type I/II IFN and vitamin-D.

- Using hypothesis generating technologies such as RNA-seq and high throughput gene expression assays such as Fluidigm, additional novel gene targets may be identified to be tease out the cellular mechanism involved in this pathway.

Long term, this study has laid the groundwork by establishing a set of assays and applying them to healthy human monocytes. A clear next step will be to apply these assays to the peripheral blood and tissue resident macrophages of HIV-infected and HIV-uninfected donors. Future studies into the imbalance of type I/II interferon observed during HIV/TB coinfection and its effect on the antimicrobial ability of monocyte/macrophages should control for exogenous bioavailable vitamin D and examine the potential of TLR1/2 activation using live *M. tb* as well as infectious HIV-1 *in-vitro.* This will facilitate more physiologically relevant conclusions and lead the way for confirmatory *ex vivo* studies of coinfection in primary cells from both the periphery and tissue samples (alveolar macrophages).

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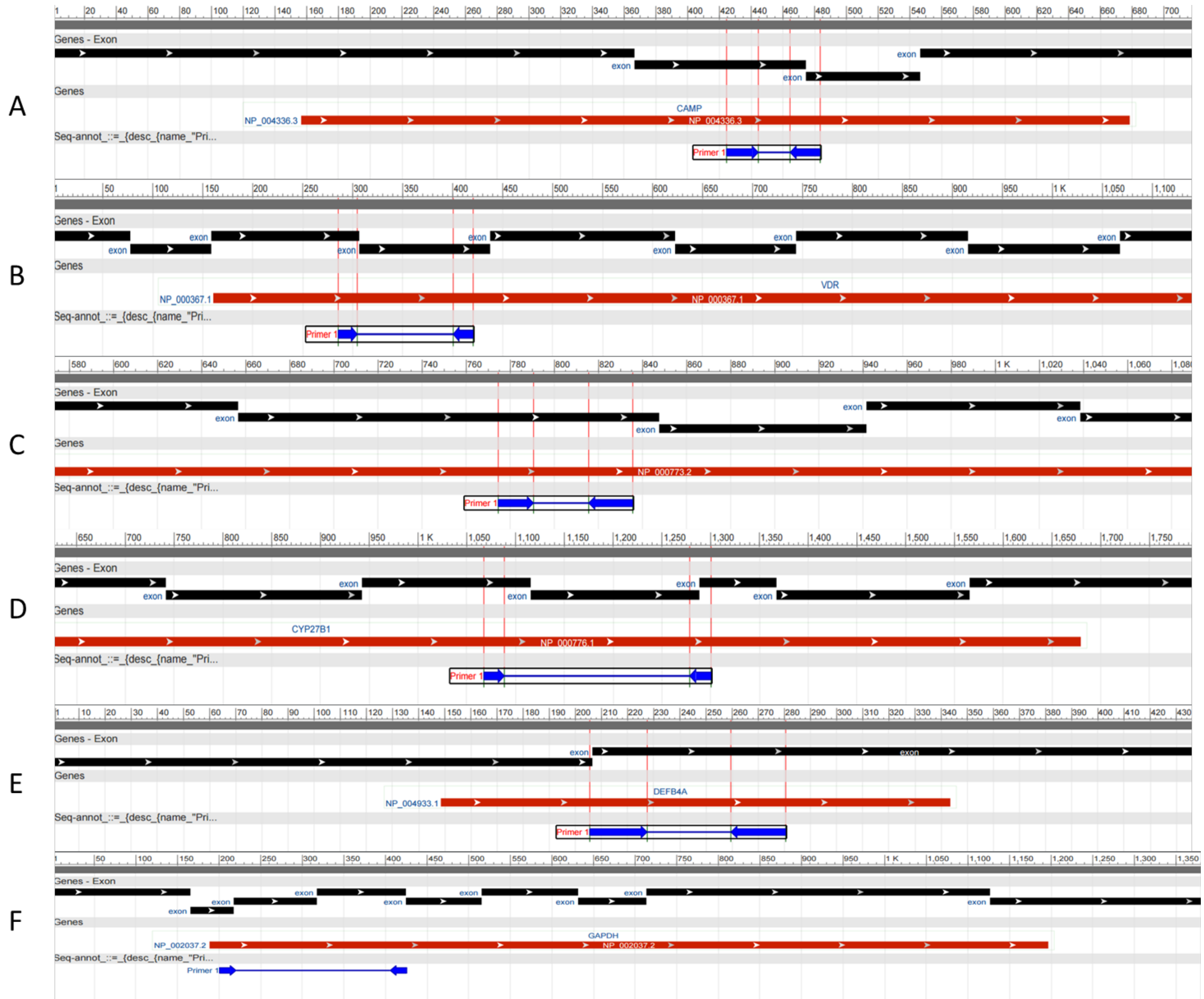
APPENDIX

Appendix 1: Conditions for the design of an Optimal Primer Pair for Specific Amplification

Parameter	Optimum Conditions
Primer Length	19 – 25 bp
Amplicon	< 400bp
Spanning	Exon – exon spanning
Stability	3’ end stable
GC Content	20 – 70%
Melting Temperature	Sense and Antisense Primers should be comparable
Variant	Account for all known variants/isoforms

Appendix 2: Primer-BLAST Alignments of Final Selected Primer-Pairs: A) CAMP, B) VDR, C) CYP24a1, D) CYP27b1, E) DEFB4 and F) GAPDH



Appendix 3: Additional Gene and Primer Information

Gene Name	*Gene Code*	*Ref Seq.*	*Gene Size (bp)*	**Exon Location**	**Primer-Pairs**	**GC %**	**Tm (°C)**	*Amplicon Size (bp)*
Cathelicidin	*CAMP*	NM_004345.4	2145	3p21.3	F 5' CCAGGACGACACAGCAGTCA 3'	60.00	62.09	59
					R 5' CTTCACCAGCCCGTCCTTC 3'	63.16	60.38	
Vitamin D Receptor	*VDR*	NM_000376.2	63495	12q13.11	F 5' AGGCTGCAAAGGCTTCTTC 3'	52.63	58.35	135
					R 5' ATGTCCACACAGCGTTTGAG 3'	50.00	58.77	
Cytochrome P450 Family 24 Subfamily A Member 1	*CYP24a1*	NM_000782.4	20529	20q13	F 5' CGCAGCGGCTGGAGAT 3'	68.75	59.45	61
					R 5' CCGTAGCCTTCTTTGCGGTA 3'	55.00	60.11	
Cytochrome P450 Family 27 Subfamily B Member 1	*CYP27b1*	NM_000785	4860	12q14.1	F 5' TCCATCCTGGGAAATGTGACA 3'	47.62	59.01	233
					R 5' ACAGGGTACAGTCTTAGCACTT 3'	45.45	58.75	
Beta-Defensin 2	*DEFB4*	NM_004942.3	2039	8p23.1	F 5' GGTGTTTTTGGTGGTATAGGCG 3'	50.00	59.84	75
					R 5' AGGGCAAAAGACTGGATGACA 3'	47.62	59.57	
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	NM_002046.5	3953	12p13	F 5' AAGGTCGGAGTCAACGGATT 3'	50.00	59.03	225
					R 5' CTCCTGGAAGATGGTGATGG 3'	55.00	57.37	
Ubiquitin C	*UBC*	NM_021009.6	2594	12q24.31	F 5' TTTCCAGAGAGCGGAACAG 3'	52.63	57.09	202
					R 5' TCACGAAGATCTGCATTGTCAAG 3'	43.48	59.32	
Beta Actin	*ACTB*	NM_001101.3	3454	7p22	F 5' GATCAAGATCATTGCTCCTCCTG 3'	47.83	58.93	121
					R 5' GCCGGACTCGTCATACTCC 3'	63.16	59.64	