Genetic factors and immune responses in chronic infectious diseases

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School of Pathology and Laboratory Medicine

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   Coordinating Supervisor Signature. ……………………………………………………………………………………………
THESIS ABSTRACT

The outcome and prognosis of chronic infectious diseases often involves genetic and immunological mechanisms which may predict susceptibility to the underlying infection or affect the consequences of infection. Here I focus my investigations on tuberculosis immune restoration disease (TB-IRD) and cytomegalovirus (CMV) disease in patients with human immunodeficiency virus (HIV) disease. Non-tuberculous mycobacterial (NTM) disease is clinically and radiographically indistinguishable from TB diagnosis and often overlooked. NTM disease diagnosis is rising worldwide due to better laboratory identification, hence I explored genetic markers as another approach to distinguish from TB or if similar genetic observations will be reproduced.

Carriage of the variant allele in TNFA-1031*C and SLC11A1 D543N*G were associated with susceptibility to developing immune restoration disease (IRD) in Cambodian HIV patients co-infected with TB. However these effects were not reflected in Indian HIV-TB patients, where the associations with susceptibility to an IRD event were increased with carriage of IL18-607*G and VDR FokI (F/f)*T. In the Appendix to Chapter 3, TB-IRD was not associated with plasma levels of Vitamin D and the VDR Fok I (F/f) allele did not predict susceptibility to succumbing to an IRD event in TB patients in Cambodia, India nor South Africa.

Caucasian NTM patients carrying IL28B-rs8099917*TG, TNFA-1031*TT, IL10-1082*AA, and IL1A+4845*AA were significantly associated with NTM disease. IL10-1082 warrants further investigation because high production of IL-10 by peripheral blood mononuclear cells (PBMC) from NTM patients has been observed previously.

Genetic polymorphisms associated with (a) susceptibility to CMV end-organ disease, and (b) predisposing low nadir CD4 T cell counts in CMV seropositive HIV-infected patients. In part (a), IL12B 3’UTR*AA and SLC11A1 D543N*AG in African Americans and Caucasian Americans carrying IL10-1082*AG and LILRB1 I142T*TT were associated with increased risk of developing CMV end-organ disease. In part (b), African Americans patients who carried DARC T-46C*AA and variant allele in CD14 C(-159)T, associated with low nadir
CD4 T cell counts. In Caucasian patients, however, associations with low nadir CD4 T cell counts were observed with carriage of TNFA-1031*TT, TNFA-308*A, IL2-330*G, CCL2-2518*GG, LILRB1 I142T*C, IL10-1082*GG and IL12B3’UTR*AC.

Immunological markers of the effects of CMV driving immune activation and inflammation were explored in a cohort of HIV patients who had been on ART for more than 12 years with good viral suppression. CMV antibody titres (reactive with lysate, gB or IE1) remain high in HIV patients who have been on ART for more than 12 years, compared to healthy CMV seropositive controls. Levels of soluble B cell activating factor (sBAFF) were higher in patients and correlated with levels of CMV antibodies, but this relationship was unclear in healthy CMV seropositive controls. In HIV patients, their CD8 T cell IFN\(\gamma\) responses against VLE peptide of the CMV IE1 protein persisted. Age was a strong factor correlated with CD57+CD45RA+CD27- expression on CD8 T-cells. This senescent phenotype also exhibited associations with antibodies against the CMV IE1 protein and CD4 T cell IFN\(\gamma\) response to CMV lysate. These results point towards an “accelerated” immune ageing effect which similar to the normal ageing process, with increased immune activation and increased levels of inflammation potentiated by periodic CMV reactivation. These observations show that CMV leaves a long-lasting effect on the immune system of HIV patients even after 12 years on ART. Extending this study in a cohort including CMV-seronegative HIV patients, CMV-DNA viral assessments and evaluation of phenotype and function of CMV-specific CD8 T cells can further elucidate how CMV reactivation promotes accelerated ageing of the immune system.

In sum, carriage of TNFA-1031*T, IL10-1082 and SLC11A1 D543N stood out the most as potential genetic markers of susceptibility in mycobacterial and CMV disease. Genetic haplotype studies looking into inflammatory markers such as tumour necrosis factor, IL10 and SLC11A1 SNPs should be further investigated in larger cohorts. Subsequent studies should investigate patterns of linkage disequilibrium to determine whether the associations observed here reflect ethnic differences in the haplotypic structure across critical genes. Functional studies investigating the effects of these SNPs may evolve into successful therapeutics for these diseases.
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General Discussion

### 1 Genetic associations with susceptibility to disease

1. **TNFA-1031*T is associated with susceptibility to chronic infectious diseases**

2. **SLC11A1 D543N*G is associated with susceptibility to TB-IRD and CMV end-organ disease**

3. **IL10-1082*A is associated with susceptibility to NTM disease, CMV end-organ disease and predisposing low nadir**

4. **IL18-607*G is associated with susceptibility to TB-IRD**

5. **VDR Fok (F/f)*T is associated with increased risk of IRD in Indian HIV-TB patients**

6. **IL28B-rs8099917 associated with susceptibility to NTM disease**

7. **IL1A+4845*AA associated with increased risk of developing NTM disease**

8. **SNPs associating with susceptibility to CMV end-organ disease and predisposition to low nadir CD4 T cell counts**

### 2 CMV Ab titres and CD8 T cell responses to CMV remain elevated and associate with greater immunosenescence in older HIV patients who began ART with very low CD4 T cell counts and achieved viral suppression

### 3 Conclusions

### 4 References
ACKNOWLEDGEMENTS

To my parents, thank you so much for the encouragement, love and support. Without your sacrifice I would never have stepped onto Australian shores for my education. To my brother and his family, thank you for words of encouragement.

To my PhD supervisors, Professor Patricia Price and Dr Silvia Lee thank you for all the time, input and encouragement, words of wisdom and believing that I can get through this. Your guidance, patience and many discussions with me all this years have enlightened me greatly. Thank you to Professor Martyn French for your suggestions when I presented data. Thank you Professor Grant Waterer for collaborating with me on the literature review. Thank you Professor Michael Lederman for collaborating with me and the positive working experience on the American project. I feel humbled walking with giants.

To the postdocs, Sonia Fernandez and Dino Tan, thank you for encouraging me to push forward when times were bleak and for all the help with Flowjo and everything else in between.

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To all patients and controls past and present, Australian and overseas, thank you very much for your contribution, for without you, there will be no research. To all collaborators local and overseas, thank you for working with me.

To my husband, Shafariiee, and my beautiful children Sophia, Zara and Daniel, this thesis brings special meaning to me. This is for you my babies – may it show you how strong a person can be and to grow up into intelligent and wonderful adults. My dear husband, thank you for putting up with me all these years, and your words of encouragement, never allowing me to give up. Thank you from the bottom of my heart.
CONTRIBUTIONS OF COLLEAGUES AND CONTRIBUTORS

The contribution of each author to each chapter contained in this thesis are listed as follows.

Chapter 2

Majority of the literature review and drafting of the manuscript was performed by myself with guidance from my supervisor Professor Patricia Price. Professor Grant Waterer contributed to the manuscript design and literature review.

Chapter 3

Experimental analysis, design and drafting of the manuscript were performed by myself with guidance from my supervisor, Professor Patricia Price. Patient recruitment and data collection in India and Cambodia was performed by Mr Manoj Kumar, in collaboration with Professor Upasna Agarwal and Professor Sarman Singh, as well as Dr Julian Elliott in collaboration with Vonthanak Saphonn and Minh Chi Vunn. Rizal Sumatoth and myself performed DNA extraction for the Cambodian cohort. Taqman genotyping of Cambodian patients and controls were performed by myself here at the Lotterywest Research Facility in RPH, Perth. Taqman genotyping of Indian patients and controls were performed by Mr Manoj Kumar and myself here at the above site. I performed the univariate statistics on GraphPad Prism and Professor Sally Burrows performed the multivariate statistics on Stata software.

Chapter 3a

Experimental design and drafting of the manuscript were done by Professor Patricia Price in collaboration with Dr Louise Haddow and Dr Philippa Easterbrook. Taqman genotyping was performed by myself here at the Lotterywest Research Facility in RPH, Perth. Plasma vitamin D assays were performed by Dr Silvia Lee. Patient recruitment from Cambodia and India have been outlined in Chapter 3 with the addition of the South African cohort in collaboration with Dr Louise Haddow and Dr Philippa Easterbrook.

Chapter 4

Experimental analysis and drafting of the manuscript were performed by myself with guidance from my supervisor, Professor Patricia Price in collaboration with Professor Grant Waterer and Dr Rachel Thomson. Experimental design and patient recruitment was
performed by Ms Shona Hendry under supervision from Professor Grant Waterer. Taqman genotyping was initially started by Ms Shona Hendry under my guidance at the Lotterywest Research Facility at RPH, Perth, and I completed the majority remaining genotyping. Professor Sally Burrows performed the multivariate analysis by logistic regression on Stata software.

Chapter 5

Experimental analysis, design and drafting of the manuscript were performed by myself with guidance from my supervisor, Professor Patricia Price in collaboration with Professor Michael Lederman and A/Prof Benigno Rodriguez from Case Western Reserve University. Mr Stephen Dando extracted the DNA and collaborated with patient data. Taqman genotyping was done by myself here at the Lotterywest Research Facility at RPH, Perth. Professor Sally Burrows performed the univariate and multivariate analysis on Stata software.

Chapter 6

Patient recruitment, laboratory experimental analysis and analysis by flow cytometry were performed by myself and Miss Jacinta Montgomery under the guidance of Dr Silvia Lee, Dr Sonia Fernandez and Dr Dino Tan. Experimental design was performed by Professor Patricia Price in collaboration with Dr David Nolan. ELISpot assays and ELISA assay for CMV lysate were performed by myself under guidance of Dr Silvia Lee. ELISA assays for CMV gB, IE, sBAFF, sCD14, total IgG were performed by Miss Samantha Brunt and sTNFR1 was by Dr Silvia Lee. Flowjo analysis was stated by Jacinta Montgomery, and final analysis was performed by under the guidance of Dr Sonia Fernandez and Dr Dino Tan. Univariate analysis was performed by myself on GraphPad Prism software and multivariate analysis was performed by Ms Samantha Brunt on Stata software. Drafting of the manuscript was done by myself under the guidance of my supervisor Professor Patricia Price.

Chapter 6a

Experimental design and drafting of the manuscript were done by Professor Patricia Price in collaboration with Dr David Nolan. Laboratory work performed for NK cells using flow cytometry was performed by Miss Jacinta Montgomery and myself. Data analysis and gating strategies were performed by Professor Patricia Price. Completion and submission of the manuscript was done by me.
STATEMENT OF CANDIDATE CONTRIBUTION

All work in this thesis was performed by the author unless otherwise stated.

---------------------------------------------------------------
Jacquita Suemarni Affandi
(PhD Candidate)

---------------------------------------------------------------
Patricia Price
(Co-ordinating supervisor)

---------------------------------------------------------------
Silvia Lee
(Co-supervisor)
The literature review presented in Chapter 2 has been published. The bibliographic details and the contribution of the student to the work are set out below.


**Student’s contribution to the work:**

Jacquita Affandi undertook literature review, created all the tables and wrote a third of the manuscript.

-----------------------------

**Patricia Price**

Co-ordinating supervisor
The data presented in Chapter 3 has been published. The bibliographic details and the contribution of the student to the work are set out below.


**Student’s contribution to the work:**

Jacquita Affandi undertook laboratory work, data analysis and wrote the manuscript.

------------------------------------

Patricia Price

Co-ordinating supervisor
The data presented in Chapter 3a has been published. The bibliographic details and the contribution of the student to the work are set out below.


Student’s contribution to the work:

Jacquita Affandi undertook the Taqman genotyping and genotyping data analysis, and wrote the genotyping data paragraph in this manuscript.

Patricia Price
Co-ordinating supervisor
The data presented in Chapter 4 has been published. The bibliographic details and the contribution of the student to the work are set out below.


**Student’s contribution to the work:**

Jacquita Affandi undertook laboratory work, data analysis and wrote the manuscript.

------------------------------------

Patricia Price

Co-ordinating supervisor
The data presented in Chapter 5 has been published. The bibliographic details and the contribution of the student to the work are set out below.


**Student’s contribution to the work:**

Jacquita Affandi undertook laboratory work, data analysis and wrote the manuscript.

------------------------------------

Patricia Price

Co-ordinating supervisor
The data presented in Chapter 6 has been published. The bibliographic details and the contribution of the student to the work are set out below.


**Student’s contribution to the work:**

Jacquita Affandi undertook laboratory work, data analysis and wrote the manuscript.

-----------------------------

**Patricia Price**

Co-ordinating supervisor
The data presented in Chapter 6a has been accepted. The bibliographic details and the contribution of the student to the work are set out below.

Jacquita S Affandi, Jacinta Montgomery, Silvia Lee and Patricia Price. **HIV patients stable on ART retain evidence of a high CMV load but changes to Natural Killer cell phenotypes reflect both HIV and CMV.** *AIDS Research and Therapy.* (accepted as of 10 November 2015)

**Student’s contribution to the work:**

Jacquita Affandi undertook laboratory work, partial data analysis, completion and submission of the manuscript.

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Patricia Price

Co-ordinating supervisor
RESEARCH OUTPUT

List of publications included in this PhD thesis

Chapter 2

Chapter 3

Chapter 3a

Chapter 4

Chapter 5

Chapter 6

Chapter 6a
7. Affandi JS, Montgomery J, Lee S and Price P. “HIV patients stable on ART retain evidence of a high CMV load but changes to Natural Killer cell phenotypes reflect both HIV and CMV”. AIDS Research and Therapy. (manuscript accepted 10 November 2015)
List of publications by Affandi JS which are not part of this PhD thesis


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>µL</td>
<td>Microlitre</td>
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<tr>
<td>AH</td>
<td>Ancestral haplotype</td>
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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
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<td>ATT</td>
<td>Anti-tubercular therapy</td>
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<tr>
<td>AU</td>
<td>Arbitrary units</td>
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<td>BAFF</td>
<td>B-cell activating factor</td>
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<td>BAT1</td>
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<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CA</td>
<td>Calcium</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CAP</td>
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<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2 (gene)</td>
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<td>Cluster of differentiation</td>
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<td>CMV seropositive</td>
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<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-responsive element-binding site</td>
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</tr>
<tr>
<td>DARC</td>
<td>Duffy antigen receptor chemokine (gene)</td>
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<td>DMSO</td>
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<td>ELISA</td>
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<td>Sulphuric acid</td>
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<td>H4TF1</td>
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<td>HLA</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>IE1</td>
<td>CMV immediate-early 1 protein</td>
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IFI16: Interferon-gamma-inducible protein 16
IFN-: Interferon
IFNGR: Interferon gamma receptor (gene)
Ig-: Immunoglobulin
IL: Interleukin
Indel: Insertion/deletion
IRD: Immune restoration disease
IRIS: Immune restoration inflammatory syndrome
IRR: Incident rate ratios
ISG: Interferon-stimulated genes
KIR: Killer immunoglobulin-like receptor (gene)
LD: Linkage disequilibrium
LILRB1: Leukocyte immunoglobulin-like receptor subfamily B member 1 (gene)
LIR1: Leukocyte immunoglobulin-like receptor 1 (protein)
LPS: Lipopolysaccharides
LTA: Lymphotoxin alpha (protein)
MAC: Mycobacterium avium complex
MAI: Mycobacterium avium-intracellulare
MBL: Mannose binding lectin (gene)
MFI: Mean fluorescence intensity
MHC: Major histocompatibility complex
mL: Millilitre
mRNA: Messenger RNA (see RNA)
MTB: Mycobacterium tuberculosis
NK: Natural killer cells
NKG2D: Natural-killer group 2, member D
NLV: NLVPMVATV peptide from CMV pp65 protein
NRAMP1: Natural resistance-associated macrophage protein 1 (gene - see SLC11A1)
NTM: Non-tuberculous mycobacteria
OI: Opportunistic infections
OR: Odds ratio
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PE: Phycoerythrin
PI: Protease inhibitor
PMA: Phorbol myristate acetate
pp65: CMV structural protein
PPD: Purified protein derivative
RA: Receptor antagonist
RNA: Ribonucleic acid
RPH: Royal Perth Hospital, Western Australia
RPMI: Roswell Park Memorial Institute medium
rs: Reference SNP
RT: Room temperature
sBAFF: Soluble BAFF (see BAFF)
<table>
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<tr>
<td>sCD14</td>
<td>Soluble cluster of differentiation 14</td>
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<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>Soluble tumour necrosis factor receptor 1</td>
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<td>Tuberculosis</td>
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<td>T_{EMRA}</td>
<td>Terminally differentiated effector memory T cells</td>
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<td>University of Western Australia</td>
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<tr>
<td>var</td>
<td>Variant</td>
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<td>VLEETSVML peptide from CMV IE1 protein (see IE1)</td>
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<td>VZV</td>
<td>Varicella zoster virus</td>
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<td>WT</td>
<td>Wild-type</td>
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Chapter 1

Introduction
It is axiomatic that the outcome of chronic diseases is affected by genetic and immunological factors. Indeed, there are several diseases originating from ubiquitous microorganisms where a majority of people have been exposed, yet only a fraction will ever succumb to the disease and manifest any signs and/or symptoms. There is a plethora of literature describing why some individuals experience autoimmune disease, but chronic infections are much more difficult to discern. Mycobacterial diseases and cytomegalovirus (CMV) infections are prime examples of this singularity. In contrast human immunodeficiency virus (HIV) is passed through contact with blood, and bodily fluids and all persons infected experience a chronic disease with debilitating outcomes including the potentiation of mycobacterial and CMV disease. Understanding the immunopathogenesis of these diseases and factors puts us in the path in identifying the hosts’ immune susceptibility and ultimately aid in elucidating new therapeutic stratagems. Here, I will explore potential genetic and immunological markers to predict susceptibility to mycobacterial and cytomegalovirus disease.

Immune activation is a hallmark of HIV infection and considered as the driving force of CD4 T cell depletion and acquired immunodeficiency syndrome (AIDS). The advent of combination antiretroviral therapy (ART) reduced mortality and morbidity of HIV infected patients although it is not a complete cure. Patients on long-term treatment are still at a higher risk of developing complications usually associated with ageing such as cardiovascular disease (CVD). This form of “accelerated immune ageing” may be attributed to opportunistic infections (OI) which are usually asymptomatic and ubiquitous, such as CMV infections. Chronic CMV reactivation potentiates exhaustion of host immune response and thus may leave an immunological “footprint”. My research is unique in addressing the long-term consequences of advanced HIV disease prior to ART in relation to CMV seropositivity and immune senescence in an older cohort.
A prime example of an OI in HIV patients is tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), and a particular burden to patients with HIV. Upon commencing ART, between 7 to 43% HIV-TB coinfected individuals experience inflammatory exacerbations and develop immune restoration disease (IRD) which has been associated with persistent immune activation. Non-tuberculous mycobacteria (NTM) is a ubiquitous microorganism usually asymptomatic in immunocompetent hosts but can lead to the development of lung disease in some individuals. It is often misdiagnosed and has similar clinical and microbiological characteristics as TB. In my thesis, NTM serves as a comparison chronic infectious respiratory and mycobacterial disease to TB, without the impairment of HIV.

Single nucleotide polymorphisms (SNP) affecting cytokines, chemokines and their receptors, as well as immune activation, may influence development of an IRD event via effects on HIV disease, on TB itself or as a response to ART. Herpesvirus infection has been associated with carriage of the variant allele in IL12B3′UTR in patients who experienced IRD.

Hence, in order to investigate genetic associations and immunological footprint of these chronic infectious diseases, I have separated my thesis into two sections. The first section of this thesis explores host genetic determinants influencing susceptibility to chronic infectious diseases whereby certain patients are more susceptible to clinical disease than others (Chapters 2-5). The second section of this thesis investigates immunological factors influencing immune ageing or senescence potentiated by CMV in a cohort of virologically suppressed HIV patients on long-term ART (Chapter 6).
1 HIV AND IMMUNE ACTIVATION

A hallmark of the pathogenesis of HIV-1 infection is the infection and depletion of CD4 T cells. ART has improved the prognosis of HIV infection, partially restoring CD4 T cell populations. However ART does not obliterate HIV or reverse the damage to the immune system. Even though viral replication is well controlled by ART in HIV patients, residual chronic immune activation still persists resulting in CD4 T cell loss. CD4 T cell depletion and AIDS is currently considered to be driven by systemic chronic immune activation thus promoting viral replication. Doitsh et al have established that pyroptosis may be the causative link between chronic inflammation and CD4 T cell depletion resulting in a pathogenic vicious cycle where dying CD4 T cells release inflammatory signals encouraging more cells to die. Interferon-gamma-inducible protein 16 (IFI16) has been identified as a host deoxyribonucleic acid (DNA) sensor required for CD4 T cell death due to abortive HIV infection, which triggers an innate immune response leading to caspase-1 activation and pyroptosis. Immune activation in HIV infection can stem from foreign antigen stimulation, including HIV, microbial products and co-infections. Type 1 interferon, microbial translocation, activated but ineffective effector T cells, dysfunctional regulatory T cells and inadequate T helper 17 cells have all been suggested to play important roles in the cycle of activation, functional exhaustion and T cell death that leads to immunodeficiency. In treated HIV infection, chronic immune activation persists and is implicated in non-infectious clinical complications of HIV that account for most of the morbidity and mortality in patients treated with ART.

The deleterious effects of “paradoxical” immune activation in HIV infection manifests as elevated markers of activation and/or apoptosis on CD8 and CD4 T cells. Although immune activation usually reflects the escalating of antiviral immunity, it can be also be predictive of an adverse prognosis for the infected patients. Direct correlation between HIV-1 disease progression and CD8 T cell activation levels can occur. HIV infection induces age-related changes to monocytes and innate immune activation in virologically suppressed HIV-infected individuals that persist despite ART.
HIV pathogenesis and disease progression has been associated with HIV-induced immune-cell activation. Hyperactivation of B cells by HIV is marked by several features, including hypergammaglobulinaemia \(^{23,24}\), increased B cell differentiation into plasmablasts \(^{24}\), and increase in frequency of B cell malignancies \(^{25}\). Several cytokines and growth factors have been suggested to directly or indirectly trigger the activation of B cells in HIV-viraemic individuals, including tumour necrosis factor (TNF) and B-cell-activating factor (BAFF) \(^{26}\). In addition, B cells activated by HIV viraemia can themselves potentiate immune activation of other cells, either by cell-cell interactions (which are favoured by the increased expression of co-stimulatory molecules such as CD80 or CD86 by B cells) or by secreting pro-inflammatory cytokines such as IL-6 and TNF. Hence, host genetic polymorphisms in cytokines such as TNF can potentiate either susceptibility or confer protection in chronic infectious diseases such as TB.
2 CHRONIC INFECTIOUS DISEASES

2.1 Immunopathogenesis of IRD in HIV-TB co-infected patients

MTB is the most common opportunistic infectious pathogen associated with HIV \(^1\) and is the leading cause of morbidity and mortality in people living with HIV/AIDS in resource-limited settings. ART has extended the lifespan of HIV patients, yet almost one in four HIV-infected patients prescribed ART experience inflammatory or cellular proliferative disease associated with a pre-existing opportunistic infection. Many of these patients have no symptoms of these underlying infections prior to ART. Symptomatic disease is most common in patients starting treatment with low CD4 T cell counts and is attributed to poor regulation of the restored immune system. These conditions were referred to as IRD and also as immune reconstitution inflammatory syndrome (IRIS) \(^1\). The most common pathogen associated with life-threatening IRD is MTB \(^27\). Paradoxical TB exacerbations in patients coinfected with HIV have a wide spectrum of presentations, ranging from mild lymph node inflammation to potentially fatal disease of the central nervous system (CNS). These exacerbations lead to TB-IRD, and there are two major presentations of these syndromes. Firstly, TB-IRD can manifest as clinical worsening of TB disease in patients in spite of favourable response to antitubercular therapy (ATT) typically arising within two months when they begin ART, defined as “paradoxical TB-associated IRIS or IRD”. Secondly, IRD may reflect subclinical TB presentation prior to ART initiation and can be defined either as ART-associated TB or “unmasking TB-associated IRIS” \(^28\). TB-IRD remains challenging to predict, diagnose and treat, hence there is an urgent need for the development of consistent laboratory biomarkers. IRD affects various sites in the body such as enlargement of the lymph nodes\(^29\) and can also commonly manifest as pulmonary \(^4\) and intra-abdominal disease \(^3\).
Currently, several risk factors have been associated with an increased risk of developing TB-IRD. High TB antigen burden\(^{30}\), a short interval between TB treatment and ART initiation\(^{6,28,31}\), and low CD4 T cell count\(^{32-34}\) have thus far been implicated. Evidence of tissue-destructive inflammation in the course of immune reconstitution points towards an augmentation of the immune response to TB bacilli or remaining antigens\(^{35,36}\). There have been reports of elevated levels of TNF\(\alpha\)^{37}, IL6\(^{38}\), IL10, MCP-1\(^{39}\) as well as other innate cytokines in the course of TB-IRD.

Retrospective genetic studies may aid in identifying mediators of IRD. SNP can intensify or reduce the effects of inflammatory cytokines, chemokine and their receptors. Genotypes and their various haplotypes, may associate with IRD either via effects on susceptibility to the underlying infection or through the degree of immunodeficiency prior to ART. Investigating different ethnicities allows observation of common disease-associated polymorphisms or haplotypes enriched from the pressures of TB in the community. Increased understanding of host responses to TB and host genetics affecting TB-IRD may guide physicians to predict or prevent IRD.

### 2.2 NTM disease

Pulmonary disease due to NTM, a pervasive environmental microorganism,\(^{40}\) is becoming more common worldwide\(^{41}\). NTM disease usually affects people over 50 years of age, so as the population ages, an increasing disease burden is inevitable. Pulmonary NTM disease caused by *Mycobacterium avium* and *Mycobacterium intracellulare* [known collectively as *Mycobacterium avium* complex (MAC) or (MAI)], is the most common in Australia\(^{41}\), with some HIV patients co-infected with NTM disease manifest as lymphadenopathy. NTM disease is often clinically and radiographically indistinguishable from TB disease. Recently, NTM infection has been implicated in the exacerbation of chronic pulmonary disorders such as chronic obstructive pulmonary disease and cystic fibrosis in immunocompromised individuals\(^{42}\). Interleukin (IL) 12 and interferon-gamma (IFN\(\gamma\)) are crucial in the host immune response to NTM infection. Defects in the IL12-IFN\(\gamma\) pathway increases susceptibility to developing NTM disease\(^{43}\).
Thus, in chapter four, I investigated if the genotypes associated with TB-IRD (Chapter 3) would occur in a cohort of HIV-negative patients who have experienced pulmonary NTM disease. Hence, I examined genetic associations in patients whose immune systems are not impaired by HIV but yet develop NTM disease.

2.3 **CMV disease**

CMV is a herpesvirus often causing asymptomatic infections in healthy individuals, but presents more severe complications in immunocompromised persons. In patients with HIV/AIDS, CMV retinitis is commonly observed. In patients initiating ART, CMV may result in IRD presenting as retinitis or uveitis, especially among HIV patients who initiate ART with CD4 T cell counts below 100 cells/µL. Most HIV-infected patients are seropositive for CMV, but only a subset develop symptomatic CMV disease. CMV can remain latent and reactivate throughout the individual’s lifespan, ageing the immune system by repeated antigenic stimulation. Chronic CMV infection has been linked with diseases of aging such as an increased risk of Alzheimer’s disease and cognitive decline.

2.3.1 **Immunological footprint of CMV and immunosenescence**

In the elderly and in those with HIV disease, chronic inflammation is associated with morbidity and mortality. Chronic viral infections such as CMV create recurrent antigenic stimulation promoting the development of an expanded population of well-differentiated, apoptosis-resistant, senescent T cells with limited proliferative potential. This culminates as an immune system with limited capacity to recognize novel antigens and subsequently prevent disease. Co-infections are common in people with HIV and appear to have a damaging clinical and immunologic effect. Hence, it is reasonable to hypothesize coinfections may contribute to the “accelerated ageing” syndrome now being observed in HIV-infected individuals. This clinical scenario likely reflects a complex condition characterized by increased burden of inflammatory diseases.
In the absence of HIV, the immune system changes with age. Optimal T cell responses are characterized by clonal expansion paralleling the generation of effector responses. Untreated HIV infection and chronological ageing are similarly associated with many T cell abnormalities. The elderly and untreated HIV-infected adults often exhibit low CD4/CD8 ratios, low naïve/memory T cell ratios, reduced T cell repertoire, and an expansion of CD57+ T cells. CD57 expression can be used to monitor proliferative history and poor proliferative capacity. Expression of CD57 on CD8 T cells has been associated with replicative senescence and antigen-induced apoptotic death. Persistent viral infections, inflammatory syndromes and ageing all induce the accumulation of highly differentiated memory T cells re-expressing CD45RA. In HIV-1 infected children, the CD45RA+CD27- phenotype on CD8 T cells correlated with CMV rather than HIV replication, as was evident in CMV seropositive healthy children. Expression of these co-stimulatory molecules together can be used to define a “senescent” phenotype on CD4 and CD8 T cells.

Very low nadir has been strongly associated with CMV end-organ disease and with elevated T-cell responses to CMV. CMV has been associated with immune ageing in elderly HIV-uninfected persons. In Chapter 6, I assessed the immunological “footprint” left by CMV in a cohort of older HIV patients who have been on ART for more than twelve years and good viral suppression for more than a year. This is to observe if immune ageing due to CMV is apparent in patients who are already immunosuppressed by HIV. HIV has been known to age the immune system prematurely, and so I explored the feasibility of CMV exacerbating this effect and leaving a “footprint” on the immune system, leading to immune senescence. Chapter 6a investigates natural killer (NK) cell phenotypes and humoral responses to CMV in the same cohort as Chapter 6.
2.4  **Host genetic predispositions – a possible contributor**

Host genetic polymorphisms contribute to variations between individuals, rate of disease progression and the degree of infectiousness as reflected by the viral load of infectious disease including the HIV. These variety of polymorphisms may also contribute to significant differences between populations with regards to HIV-1 prevalence \(^{44,45}\), as regional variations of the HIV-1 pandemic are not easily elucidated by social factors and sexual behaviour \(^{46,47}\). Undermining the importance of such polymorphisms may confound the assessment of vaccine development and therapeutic microbicides.

Genetic variances within the immune system can influence the immunopathogenesis and thus morbidity of infectious diseases. Ethnicity also plays a part as different genetic associations exist in differing inheritance of polymorphisms. A prime example of this is the Duffy antigen receptor chemokine (DARC) SNP. Carriage of the variant allele on DARC T-46C, conferring a DARC-negative phenotype, has been associated with protection among individuals of African descent against *Plasmodium vivax* malaria infection \(^{48}\). This SNP has also been associated with susceptibility to HIV infection and rate of progression to AIDS \(^{49}\). Genetic polymorphisms predisposing the carrier to chronic infectious diseases will be discussed further in Chapters 2, 3, 4 and 5.
3 HYPOTHESIS AND AIMS OF THIS THESIS

Host genetic polymorphisms can predispose carriers to mycobacterial and CMV disease, and that constant CMV reactivation accelerates immune ageing and leaves potential “footprints” on the immune system.

Thus, the aim of this thesis was to (a) investigate host genetic associations which can help predict susceptibility and elucidate the immunopathogenesis of chronic infectious diseases, and (b) understanding possible long-term effects of ART and CMV-seropositivity on the immune system, identify possible biomarkers and how it may promote accelerated ageing.

In order to resolve part (a), I undertook a literature review to aid in the identification of immune-related genetic polymorphisms which have been associated with various chronic respiratory diseases such as community-acquired pneumonia (CAP), TB and NTM (Chapter 2). Henceforth, I focused on investigating several SNPs relating to inflammation and immune activation, and identify trends and associations with susceptibility to an increased risk of developing an IRD event in HIV patients co-infected with TB from two countries, namely Cambodia and India (Chapter 3). If a significant association is similar in two ethnicities, it indicates the biological importance of that SNP. Opposing observations between ethnicities supports the hypothesis that the effect is random or that the haplotypic associations around the SNP differ with ethnicity.

A further collaboration allowed an investigation to determine if plasma vitamin D levels and genetic polymorphisms in the vitamin D receptor can predict susceptibility to an IRD event in HIV patients co-infected with TB with various ethnicities, namely Cambodians, Indians and South Africans (Chapter 3a).
With the results from Chapter 3 and 3a, I then investigated whether the same SNPs showing trends associating with TB-IRD will be reflected in another cohort of patients with mycobacterial disease who are not infected with HIV disease (Chapter 4). As TB-IRD is a disease associated with low CD4 T cell counts, I shifted my focus to CMV disease, another opportunistic infection closely associated with HIV, often marked by very low nadir CD4 T cell counts. Thus, I examined if immune-related SNPs show associations with susceptibility to an increased risk of developing CMV end-organ disease and predisposition to low nadir CD4 T cell counts (Chapter 5).

In order to further my understanding of CMV disease, I then sought to investigate part (b) of my aims. HIV patients and healthy controls were recruited to observe whether CMV co-infection in HIV-infected individuals potentiates alterations within the T cell compartment and thus promotes accelerated ageing in HIV patients stable on long-term ART. Senescent markers such as the presence of CD57, CD45RA and the absence of CD27 on CD4 and CD8 T cells were studied, with comparisons made within the cohort. Antibodies to CMV proteins, IFNγ responses to CMV antigens, immune activation markers such as soluble tumour necrosis factor receptor 1 (sTNFR1), markers of B cell activation, and natural killer cells’ functions were investigated, in order to identify possible biomarkers of the “footprint” of CMV disease on the immune system (Chapters 6 and 6a).
4 REFERENCES


Chapter 2

Can immunogenetics illuminate the diverse manifestations of respiratory infections?


This chapter is presented in the form accepted for publication and constitutes pages 18-33 of this thesis.

Chapter outline:

This chapter is a literature review describing immune-related genes that can explicate the immunopathogenesis of commonly known chronic respiratory diseases such as CAP, TB and NTM.
Can immunogenetics illuminate the diverse manifestations of respiratory infections?

Jacquita S. Affandi, Patricia Price and Grant Waterer

Abstract: Improved technologies for high-throughput genotyping and the establishment of well-defined cohorts prompted hope that polymorphisms would be discovered that define a patients’ risk of respiratory disease or aid in diagnosis. Genetic pitfalls encountered in this quest include genotyping errors, ethnic differences and linkage dysequilibrium. Differences in the definition of the disease phenotype also create discrepancies, so immunogenetic testing has not yet reached the clinic. However, associations between a polymorphism and a disease phenotype place the gene or one in linkage dysequilibrium on the path to the disease. Here we review studies of immune-related genes that are illuminating the immunopathogenesis of community-acquired pneumonia and mycobacterial infections.

Keywords: immunogenetics, respiratory infections, tuberculosis, mycobacteria, community acquired pneumonia

Introduction
There is no question that genetic differences have an impact on the risk of developing or dying from infection. The familial risk of death from infection may be greater than that from cancer or cardiovascular disease [Sorensen et al. 1988]. This fits the reality that infectious diseases were the largest cause of death during our evolution. Variation in individual outcomes following exposure to a respiratory pathogen and differential susceptibility to pulmonary infections reflect the genetic diversity in immune responses that have evolved in response to infectious challenges [Burgner et al. 2006]. Indeed, immune-related genes are numerous and diverse [Murphy, 1993].

While pharmacogenetic studies have advanced many fields (notably oncology), diverse and extensive explorations of genetic influences on infectious diseases have provided no real clinical applications. Rare Mendelian immunologic deficiencies affect immunoglobulins, complement components, rearrangement of the T-cell receptor, a few cytokines and some cytokine receptors producing characteristic and severe phenotypes [Quintana-Murci et al. 2007]. However, the complexity and redundancy in immunological pathways allows multiple genetic influences to affect outcome, depending on the pathogen and environmental factors. Moreover, most genetic variations are single nucleotide polymorphisms (SNP) which do not abrogate production of the protein or generate a dysfunctional protein. Rather they lie in putative regulatory areas and affect the rate of transcription or the stability of the mRNA product, creating a more subtle phenotype.

Common limitations with genetic studies in pulmonary infection
Several problems are common in gene association studies and limit interpretation or application of the findings. These need to be addressed to minimize spurious associations.

(1) Linkage dysequilibrium (LD) refers to the coinheritance of polymorphisms across segments of DNA that can encompass many genes (Figure 1), creating conserved haplotypes. Without detailed mapping of the DNA region around the polymorphism(s), observed associations may actually reflect a coinherited polymorphism in the same gene.
or one close by. Examples are evident in our discussion of tumour necrosis factor (TNF) and lymphotoxin alpha (LTA).

(2) Ethnic populations have different frequencies of polymorphisms and different haplotypes. Some haplotypes are restricted to specific populations. This may reflect a recent polymorphism or survival of a particular founding population [Valente et al. 2009]. It is critical that cases and controls are drawn from the same racial (e.g. genetic) background.

(3) Replication of results may be hampered by demographic differences in the populations studied. Age (paediatric versus adult versus elderly), comorbidities, severity of illness and differences in the pathogen mix can influence which genes have an impact upon outcome.

(4) Even a small number of genotyping errors can alter the results of a study, especially when the number of outcomes is relatively small. Many studies do not report quality assurance protocols to ensure that genotyping errors were minimized. Quality assurance protocols should address analytical discrepancies due to small study cohorts, nonuniform experimental and/or statistical methods and poor control of confounding methods. Deviations from Hardy–Weinberg equilibrium are often ignored, but may flag genotyping errors.

It is worthwhile to define the criteria that must be satisfied to consider a polymorphism contributes directly to genetic risk. These include:

(a) extensive validation of the association in more than one independent cohort representative of different ethnic populations across the globe;
(b) evidence of altered production or function of the protein product of the gene carrying the polymorphism;
(c) demonstration that the association does not reflect LD with other candidates.

**Genetic susceptibility to community-acquired pneumonia**

Community-acquired pneumonia (CAP) can be defined as an acute infection of the lower respiratory tract occurring in a patient who has not resided in a hospital or healthcare facility in the previous 14 days [Bartlett, 2000]. It is a common and potentially life-threatening illness. It is caused by a range of organisms, most commonly *Streptococcus pneumoniae, Mycoplasma pneumoniae, Chlamydia pneumoniae* or respiratory viruses [Johnson et al. 2002]. Pathophysiology and risk factors are still poorly understood. Coexisting illnesses can increase the risk of CAP, although the causal mechanisms are unclear, but patients with...
no underlying health problems can die from pneumonia. Although old age is a risk factor, more than half of deaths from bacteremic pneumococcal pneumonia occur between 18 and 65 years of age [Feikin et al. 2000].

Understanding the effect of variant SNP or haplotype on the amount or function of an encoded protein should illuminate its role in the pathogenesis of CAP. Genetic studies may identify aspects of pathophysiology that are not apparent from traditional laboratory studies [Wunderink and Watterer, 2004].

Many genes have been studied to identify polymorphisms that affect CAP. While even more have been studied in relation to sepsis, the findings cannot be generalized to pneumonia [Russell et al. 2010]. Hence, we limit this review to pneumonia. Only mannose-binding lectin (MBL, encoded by the MBL2 gene) and the IgG2 Fc gamma receptor IIa (FcγRIIa also known as CD32, encoded by the FCGR2 gene) currently meet the criteria established above. We discuss these first. Other polymorphisms are grouped by their place in the inflammatory response (see Table 1) and discussed in the review of candidate SNPs section.

MBL is a soluble pattern-recognition molecule that binds micro-organisms and has independent ability to activate the complement system. Several polymorphisms within the gene or in the promoter region influence serum concentrations of MBL and consequently affect the outcome of many types of infections [Garred et al. 2006]. A meta-analysis of three case–control studies associated homozygosity and compound heterozygosity for MBL2 variant alleles with an increased risk of invasive pneumococcal disease in homogenous white populations from Britain [Roy et al. 2002], Denmark [Kronborg et al. 2002] and Belgium [Moens et al. 2006a] (see Panel 1 of Table 1). However, when these studies were analysed separately, the large British study overwhelmed the smaller negative studies from Denmark and Belgium. These studies differed in the age of patients, cohort size and age group. In a subsequent multivariate analysis, carriage of the MBL2 variant alleles was associated with the development of the most severe forms of sepsis, acute respiratory failure, multiorgan dysfunction syndrome, intensive care unit admission and death in a large cohort of Spanish patients with CAP [Garcia-Laorden et al. 2008].

Three major classes of immunoglobulin G receptors are expressed on human leukocytes: FcγRI (CD64); FcγRII (CD32); and FcγRIII (CD16). Human FcγRIIa (encoded by FCGR2) has two codominantly expressed isoforms which differ by one amino acid arginine (R, Arg) to histidine (H, His) at position 131 which results from a single base substitution. FcγRIIa-R131 protein (encoded by C allele at FCGR2 Arg131His (rs1801274)) is associated with decreased binding of human IgG2 and IgG3 compared with FcγRIIa-H131 protein (encoded by T allele) [Parren et al. 1992]. Two studies suggest that homozygosity for the C allele at FCGR2 Arg131His is a risk factor for bacteremic pneumococcal pneumonia in paediatric patients [Yuan et al. 2008; Yuan and Sullivan, 2009], while another found a positive association with mortality and length of hospital stay [Endeman et al. 2009]. These results were not replicated by another study [Moens et al. 2006b], but this had only 55 patients.

LD with polymorphisms in IL10 in the same region of chromosome 1 complicates the assessment of the FCGR2 Arg131His polymorphism [van der Pol et al. 2001]. No published study of pneumonia has performed haplotype mapping across the entire FCGR2 and IL10 region so critical polymorphisms have not been identified, although the biological basis for a role for FcγRIIa is relatively sound. FCGR2 genotypes also exhibit ethnic variation. Asian populations (Chinese, Japanese and Vietnamese) have a lower frequency of homozygous C allele at FCGR2 Arg131His, and a high frequency for homozygous T allele at FCGR2 Arg131His as compared with Whites and Africans. Ethnic variation of FCGR2 may explain why certain diseases vary between ethnic populations, but this needs further study [Yuan and Sullivan, 2005].

Genetic studies of mycobacterial disease

*Mycobacterium tuberculosis* (MTB) causes tuberculosis (TB) and is a major cause of illness and deaths, but some individuals never develop disease despite a lifetime spent in regions of Asia and Africa where the infection is endemic. Similarly it is likely that everyone in the world is exposed to nontuberculous mycobacteria (NTM; *Mycobacterium kansasii* and *Mycobacterium avium-intracellularare*) but most people never display signs or symptoms of infection. In countries where molecular diagnosis of chronic infections is standard practice, children with defined
genetic defects are severely affected by NTM [Remus et al. 2001] and some people, notably older ladies with no identifiable risk factor, experience chronic pulmonary NTM disease [Han et al. 2005]. Subtle changes in immune responses may confer an increased risk in the context of normal age-associated changes in immunocompetence.

NTM disease and TB are more common and severe in patients coinfected with HIV. In addition, immune reconstitution on antiretroviral therapy (ART) invokes an exacerbation of symptoms in a subset of patients (immune restoration disease [IRD]) [Price et al. 2009]. Susceptibility may reflect factors that influence mycobacterial disease in patients without HIV, as well as genetic determinants of HIV-associated immune dysfunction.

Clearance of mycobacteria requires an effective cell-mediated immune response [Hill, 1998]. Interleukin (IL)-12 drives production of interferon gamma (IFNγ) from natural killer (NK) cells and antigen-specific Th1 cells. The activation of infected macrophages by IFNγ, in synergy

Table 1. Associations between single nucleotide polymorphisms (SNPs) in immune-related genes and community-acquired pneumonia (CAP).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Association with CAP</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Mannose-binding lectin (MBL) mediates recognition of bacteria by monocytes/macrophage</strong></td>
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<tr>
<td>MBL2 Gly55Asp [G/A] [rs1800450] <strong>B allele</strong></td>
<td>Carriage of variant alleles associated with increased risk of invasive pneumococcal disease in a combined White Danish, British and Belgian cohort</td>
<td>[Moens et al. 2006a; Kronborg and Garred, 2002; Roy et al. 2002]</td>
</tr>
<tr>
<td>MBL2 Gly576Gui [A/G] [rs1800451] <strong>C allele</strong></td>
<td>Carriage of variant alleles in a multivariate analysis was associated with higher severity and fatal outcome of White Spanish cohort</td>
<td>[García-Laorden et al. 2008]</td>
</tr>
<tr>
<td>MBL2 Arg52Cys [C/T] [rs5030737] <strong>D allele</strong></td>
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</table>

| **Fcγ receptor II is critical for the opsonization of bacteria**                       |
| FcγR2 Arg131His [C/T] [rs1801274]                                                    | Homozygous allele 1 is a risk factor for bacteremic pneumococcal pneumonia | [Yuan et al. 2008; Yuan and Sullivan, 2005] |
| Homozygous allele 1 is associated with severity of CAP and is more frequent in CAP caused by H. influenzae. No association with CAP in a White Belgian cohort | [Endeman et al. 2009] |

| **Toll-like receptors (TLRs)**                                                        |
| TLR2-16934 [T/A] [rs4696480]                                                         | Homozygous allele 2 associated with increased risk of pneumonia           | [Sutherland et al. 2005] |
| TLR2 Arg677Trp [C/T] [rs5743706]                                                     | Carriage of allele 2 associated with Gram-negative sepsis                  | [Lorenz et al. 2002] |
| TLR2 Arg753Gln [G/A] [rs5743708]                                                     |                                                                           | |
| TLR4 Asp299Gly [A/G] [rs4968790]                                                     |                                                                           | |
| TLR5 Arg392Stopcodon [1174, [C/T]]                                                   | Carriage of allele 2 in individual SNPs, and haplotypes TLR5 1174*2-1175*1 and TLR5 1174*1-1175*2, associated with increased risk of Legionella pneumophila infection | [Hawn et al. 2003] |

| **Tumour necrosis factor and IL-1 mediate fever and inflammation associated with CAP** |
| TNF-308 [G/A] [rs1800629]                                                           | No association with CAP                                                   | [Sole-Violan et al. 2009; Kinder et al. 2007; Waterer et al. 2001b] |
| TNF-238 [G/A] [rs361525]                                                            | Carriage of allele 2 associated with increased risk of adverse outcome of pneumonia | [Kinder et al. 2007] |
| TNF-308 [G/A] [rs1800629]                                                           | TNF308*2-238*2 haplotype is associated with being a better predictor of higher mortality | [Henckaerts et al. 2009; Pappachan et al. 2009] |
| TNF-238 [G/A] [rs361525]                                                            |                                                                           | |
| TNFRSF1B+476 [G/T] [Met196Arg, rs1061622]                                            | Heterozygosity is protective against the outcome of CAP                   | [Sole-Violan et al. 2009] |

Notation of alleles is gene-nucleotide position (allele 1/allele 2), with allele 1 being the most frequent in the population (wild-type). Amino acid changes are presented where they arise.
with TNFα, is a major effector mechanism. In resting macrophages, mycobacteria reside within modified phagosomes that intersect with the host cell’s recycling endosomal network, actively blocking encounters with lysosomes. Activation removes the block in lysosome fusion and exposes bacteria to a proton-rich lysosome, where hydrolases, reactive nitrogen intermediates and reactive oxygen intermediates kill replicating bacteria. Clearance of all cells harbouring latent infections is mediated by cytolytic CD8 T cells. This is also promoted by Th1 cytokines. The importance of TNF is illustrated by evidence that TNF inhibitors administered as therapy for Crohn’s disease or rheumatoid arthritis promote TB [Desai and Furst, 2006]. All aspects of this pathway reflect candidate genes with potential to affect the outcome of mycobacterial infection.

**Tuberculosis**

Host genotype is likely to be an important determinant of susceptibility to TB, because monozygotic twins have a higher concordance than dizygotic twins [Comstock, 1978]. The infection is airborne and endemic in many countries. Treatment is protracted and relapse is common. The World Health Organization is extending treatment and prevention strategies with a goal of halving 1990 prevalence and death rates by 2015. Despite impressive progress in many countries, this goal is unlikely to be met for the world as a whole. Several recombinant vaccines are now being trialled [Scriba et al. 2010], but their evaluation requires an understanding of the immune mechanisms critical for protection. Genetic studies can reveal the relative importance of immunological pathways identified in animal studies. Published associations are summarized in Table 2. Candidate genes are discussed in the review of candidate SNPs section.

**Disease associated with NTM in patients not affected by HIV**

A number of polymorphisms and mutations are associated with recurrent NTM lymphadenitis in children. Most affect the IFNγ–IL-12 axis [Fernando and Britton, 2006; van de Vosse et al. 2004]. Adults with pulmonary NTM disease do not display these associations, possibly because defects in the IFNγ–IL-12 pathway generate marked immunodeficiency and an early onset of disease. NRAMP1 polymorphisms may associate with adult pulmonary NTM disease (as in mice) [Koh et al. 2005], but this was not uniformly observed [Huang et al. 1998].

Deleterious mutations in the IFNGR1 and IFNGR2 encoding for the subunits of the receptor for IFNγ (IFNγR1 and IFNγR2) are common in individuals with recurrent severe infections with otherwise weakly pathogenic mycobacteria, such as NTM or bacille Calmette–Guerin (BCG) attenuated vaccines, and with *Salmonella* species [Ottenhoff et al. 2002]. Complete IFNγR1 deficiency is characterized by infections with NTM at an early age and impaired granuloma formation. Partial IFNγR1 deficiency associates with a later onset of infection and well-differentiated granulomas [Doffinger et al. 2006]. For example, partial deficiency in IFNγR1 can be caused by homozygous amino acid substitution in the extracellular domain resulting in diminished IFNγ binding and/or signalling or by heterozygous frameshift deletions yielding IFNγR1 molecules that lack a cytoplasmic tail critical for cellular signalling. Other SNP with potential to affect NTM disease are summarized in Table 3. Candidate SNP are discussed in the review of candidate SNPs section.

**Mycobacterial immune restoration disease**

A proportion of HIV-infected patients treated with combination ART experience atypical presentations of diseases arising from infection by pre-existing (often quiescent) opportunistic pathogens. These can occur despite favourable virological responses and increases in CD4+ T-cell counts. Illness associated with cytomegalovirus (CMV), *Mycobacterium avium* complex (MAC), MTB, varicella zoster virus (VZV) or herpes simplex virus (HSV) and hepatitis B or C virus (HCV) have been described by us and others (reviewed by Price et al. [2009]). We introduced the term IRD to describe these conditions. Whilst IRDs may resolve spontaneously, some result in significant morbidity or mortality. This includes TB, which commonly presents as an IRD in countries where TB is endemic and ART is only now becoming available.

TB-IRD can affect many sites in the body (reviewed by Price et al. [2009]) and clinical manifestations depend on the site of infection. Pulmonary and intra-abdominal diseases are common, including worsening pulmonary infiltrates, new pleural effusions, increased or new lymphadenopathy. Many patients initially treated for pulmonary TB develop additional manifestations of IRD at extrapulmonary sites [Colebunders et al. 2006]. IRD in a
<table>
<thead>
<tr>
<th>SNP</th>
<th>Association with mycobacterial disease</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Toll-like receptor 2 (TLR2) is critical for the recognition of mycobacteria by antigen presenting cells (APC)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2 Arg753Gln (G/A)</td>
<td>Carriage of allele 2 associated with TB in Turkish patients</td>
<td>[Ogus et al. 2004]</td>
</tr>
<tr>
<td>TLR2 intron II (GT) [n] microsatellite</td>
<td>Shorter (n \leq 16) GT repeats in intron II of the human TLR2 gene associated with TB disease in a Korean cohort</td>
<td>[Yim et al. 2006]</td>
</tr>
<tr>
<td><strong>Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN, encoded by the CD209 gene) is a cell surface receptor for mycobacteria on DCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD209 A-336G (A/G)</td>
<td>Carriage of allele 2 was protective from TB in Gambians but outcome was variable in other African populations and homozygous allele 2 promoted lung caviation</td>
<td>[Vannberg et al. 2008]</td>
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<tr>
<td></td>
<td>Carriage of allele 1 was protective from TB in South Africans, but allele 2 of CD209-871 had a clearer protective effect</td>
<td>[Barreiro et al. 2006]</td>
</tr>
<tr>
<td></td>
<td>No effect on TB in Colombians</td>
<td>[Gomez et al. 2006a]</td>
</tr>
<tr>
<td></td>
<td>No effect on TB in Tunisians</td>
<td>[Ben-Ali et al. 2007]</td>
</tr>
<tr>
<td><strong>Cluster of differentiation 14 (CD14) is involved in the recognition of lipopolysaccharides (LPS) and bacterial cell wall components by macrophages</strong></td>
<td></td>
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<tr>
<td>CD14 C-159T (C/T)</td>
<td>Homozygous allele 2 associated with TB in Mexicans</td>
<td>[Rosas-Taraco et al. 2007]</td>
</tr>
<tr>
<td></td>
<td>No association with pulmonary TB in Colombians</td>
<td>[Pacheco et al. 2004]</td>
</tr>
<tr>
<td><strong>Tumour necrosis factor and IL-1 mediate fever and inflammation associated with TB</strong></td>
<td></td>
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</tr>
<tr>
<td>TNFA-308 (G/A) [rs1800629]</td>
<td>No association with TB in South Indian, Korean, Thai or Turkish patients</td>
<td>[Ates et al. 2008; Oh et al. 2007; Vejbaesya et al. 2007; Selvaraj et al. 2001]</td>
</tr>
<tr>
<td>TNFA-238 (G/A) [rs361525]</td>
<td>TNF-308 allele 1 associated with TB, whilst allele 2 associated with several autoimmune diseases in Colombians</td>
<td>[Correa et al. 2005]</td>
</tr>
<tr>
<td>TNFA-376 (G/A) [rs1800750]</td>
<td>TNF-238 (allele 2 was protective for autoimmunity but associated with susceptibility for TB)</td>
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<tr>
<td>TNFA +488 (G/A)</td>
<td>The haplotype TNF-308<em>2-238</em>1 was protective against TB but carried susceptibility for autoimmune diseases</td>
<td></td>
</tr>
<tr>
<td>TNFA-308 (G/A) [rs1800629]</td>
<td>No association with pulmonary TB in HIV negative Cambodian patients</td>
<td>[Delgado et al. 2002]</td>
</tr>
<tr>
<td>TNF-10311 (T/C) [rs7999964]</td>
<td>Carriage of allele 2 associated with protection in Colombians</td>
<td>[Gomez et al. 2006b]</td>
</tr>
<tr>
<td>IL1B +3953 (C/T) [rs1143634]</td>
<td>Microsatellite alleles influence TB in Gambians. No effect of IL10 or IL1B SNP</td>
<td>[Bellamy et al. 1998a]</td>
</tr>
<tr>
<td>IL1 receptor antagonist, IL1, IL10</td>
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<tr>
<td><strong>IL-12 drives a Th1 response; SNP may affect expression of the cytokine and its receptor</strong></td>
<td></td>
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<tr>
<td>IL12B intron 2 [ATT] [n] [ATT] [m] repeat microsatellite marker [DSS2941]</td>
<td>Homozygous allele 1 associated with increased risk of developing TB in Hong Kong Chinese</td>
<td>[Tso et al. 2004]</td>
</tr>
<tr>
<td>IL12B 3'UTR / IL12B +1188 (A/C) [rs3212227]</td>
<td>No association with pulmonary TB in South Indian patients</td>
<td>[Prabhu Anand et al. 2007]</td>
</tr>
<tr>
<td></td>
<td>No association with susceptibility to TB, but homozygous allele 2 linked with increased levels of IL-12p40 (South Indian)</td>
<td>[Selvaraj et al. 2008b]</td>
</tr>
<tr>
<td></td>
<td>Carriage of allele 2 associated with TB in Hong Kong Chinese</td>
<td>[Tso et al. 2004]</td>
</tr>
<tr>
<td></td>
<td>No association with TB (African American and whites)</td>
<td>[Ma et al. 2003]</td>
</tr>
<tr>
<td>SNP</td>
<td>Description</td>
<td>Ethnicity/Population</td>
</tr>
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<td>----------------------------</td>
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<td>---------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IL12RB1 -2 (C/T)</td>
<td>Carriage of allele 2 associated with TB in Moroccans</td>
<td>[Remus et al. 2004]</td>
</tr>
<tr>
<td>IL12RB1 +467 G/A</td>
<td>No association with pulmonary TB in Indonesians</td>
<td>[Sahiratmadja et al. 2007]</td>
</tr>
<tr>
<td>+641 A/G (Q214R)</td>
<td>The 214<em>2-365</em>2-378*2 haplotype associated with susceptibility to TB in Japanese patients</td>
<td>[Akahoshi et al. 2003]</td>
</tr>
<tr>
<td>+705 A/G (Q214R)</td>
<td>Carriage of allele 2 of the +641, +1094 and 1132 SNPs associated with pulmonary TB susceptibility and severity in Japanese patients</td>
<td>[Kusuhara et al. 2007]</td>
</tr>
<tr>
<td>+1094 T/C (M365T)</td>
<td>The +705, +1158, +1196, +1637 and +1664 SNPs were not associated with TB in Koreans</td>
<td>[Lee et al. 2005]</td>
</tr>
<tr>
<td>+1132 C/G (G378R)</td>
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<tr>
<td>+1158 T/C (M365T)</td>
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<td></td>
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<tr>
<td>+1196 G/C (G378R)</td>
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<tr>
<td>+1312 C/T</td>
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<td>+1573 G/A</td>
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<tr>
<td>+1637 G/A (A525T)</td>
<td></td>
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</tr>
<tr>
<td>+1664 C/T (P534S)</td>
<td></td>
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<tr>
<td>+1781 G/A</td>
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</tr>
</tbody>
</table>

**IL10 inhibits TNF and Th1 (IFNγ) responses to TB**

- IL10-1082 (A/G) rs1800896: Carriage of allele 2 was associated with TB in Turkish patients. No effects with IL10-819 (C/T) or IL10-592 (C/A)
  - [Ates et al. 2008]

**IFNγ mediates aTh1 response activating monocytes to kill engulfed mycobacteria**

- IFNG +874 (T/A) rs2430561: Homozygous allele 2 associated with increased risk of developing TB in a Spanish cohort
  - [Lopez-Maderuelo et al. 2003]

- IFNG-1616 (G/A): Homozygous allele 1 of IFNG-1616, homozygous allele 1 of IFNG +3234 and homozygous allele 2 IFNGR1-56 associated with susceptibility to pulmonary TB in West Africans
  - [Cooke et al. 2006]

**IL-2 expands a T-cell response**

- IL2-330 (G/T) rs2069762: Carriage of allele 2 associated with protection from pulmonary TB in South Indians
  - [Selvaraj et al. 2008b]

**Low levels of circulating 1,25 (OH)2-Vit D3 have been associated with susceptibility to TB**

- VDR Taq1 (T/C) rs731236: Homozygous allele 1 associated with protection from TB in Gambians
  - [Bellamy et al. 1999]

- VDR Cdx-2 (G/A): Homozygous allele 2 phenotype associated with protection against TB in Indians
  - [Selvaraj et al. 2008a]

- VDR Fok1 (C/T), VDR Bsm1 (T/C), VDR Apa1 (T/G), VDR Taq1 (T/C): The Fok1*1-Bsm1*2-Apa1*1-Taq1*1 haplotype is significantly associated with protection from TB in Venda South Africans
  - [Lombard et al. 2006]
## Table 2. Continued.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Association with mycobacterial disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human leukocyte antigen (HLA)</strong></td>
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<tr>
<td>DRB1<em>1302-DQB1</em>0602/3; DRB1<em>1101-1121-DQB1</em>0503</td>
<td>Significantly associated with TB in Venda South Africans</td>
<td>[Lombard et al. 2006]</td>
</tr>
<tr>
<td>DRB1<em>1101-1121-DQB1</em>0304; DRB1<em>1101-1121-DQB1</em>05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*0503</td>
<td></td>
<td></td>
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<tr>
<td>DQB1*0502</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQA1<em>0601, DQB1</em>0301</td>
<td></td>
<td></td>
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<tr>
<td>DRB1*16</td>
<td></td>
<td></td>
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<tr>
<td>DRB1*13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRB1<em>1601-DQB1</em>05; DRB1<em>1601-DQB1</em>0502; DRB1<em>04-DQB1</em>03; DRB1<em>14-DQB1</em>05</td>
<td>Associated with increased risk of development of TB in a Polish cohort</td>
<td>[Dubaniewicz et al. 2005]</td>
</tr>
<tr>
<td>DQB1<em>0201; DQB1</em>0301</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1<em>0401/02; DQB1</em>0503; DQB1*0601/02/03</td>
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<tr>
<td><strong>NRAMP1 (also known as SLC11A1) encodes a divalent cation transporter which removes Fe^{2+} from phagosomes</strong></td>
<td></td>
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<tr>
<td>NRAMP1 D543N G/A (rs17235409)</td>
<td>Carriage of allele 2 associated with susceptibility to pulmonary TB in females and patients under 65 years in a Chinese cohort</td>
<td>[Leung et al. 2007]</td>
</tr>
<tr>
<td></td>
<td>Heterozygosity associated with severe forms of pulmonary TB but not susceptibility to TB in Chinese</td>
<td>[Zhang et al. 2005]</td>
</tr>
<tr>
<td></td>
<td>Carriage of allele 2 associated with TB in Gambian, Japanese and Cambodian cohorts</td>
<td>[Delgado et al. 2002; Gao et al. 2000; Bellamy et al. 1998b]</td>
</tr>
<tr>
<td></td>
<td>Homozygous allele 2 associated with susceptibility to pulmonary TB in a Chinese Han cohort</td>
<td>[Liu et al. 2004]</td>
</tr>
<tr>
<td>NRAMP1 3’UTR [TGTG+/−deletion]</td>
<td>carriage of allele 2 associated with tuberculous pleurisy in Koreans</td>
<td>[Kim et al. 2003b]</td>
</tr>
<tr>
<td></td>
<td>Not associated with microscopy-positive TB in a Danish cohort</td>
<td>[Soborg et al. 2002]</td>
</tr>
<tr>
<td></td>
<td>Heterozygosity associated with susceptibility to pulmonary TB in a Chinese Han cohort</td>
<td>[Liu et al. 2004]</td>
</tr>
<tr>
<td>NRAMP1 INT4 (G/C)</td>
<td>Heterozygosity associated with susceptibility to TB in Gambians</td>
<td>[Bellamy et al. 1998b]</td>
</tr>
<tr>
<td></td>
<td>Heterozygosity associated with severe forms of pulmonary TB but not susceptibility to TB in Chinese patients</td>
<td>[Zhang et al. 2005]</td>
</tr>
<tr>
<td></td>
<td>Heterozygosity associated with increased mycobacterial replication rather than susceptibility to TB, thus increasing risk of severe disease in a Danish cohort</td>
<td>[Zhang et al. 2005; Kim et al. 2003a; Soborg et al. 2002]</td>
</tr>
<tr>
<td>NRAMP1 5’CA)n-allele 1 [199bp]</td>
<td>Carriage of allele 2 associated with tuberculous pleurisy in Koreans</td>
<td>[Kim et al. 2003a]</td>
</tr>
<tr>
<td>-allele 2 [201bp]</td>
<td>Carriage of allele 2 or 3 associated with increased mycobacterial replication rather than susceptibility to TB, thus increasing risk of severe disease in a Danish cohort</td>
<td>[Zhang et al. 2005; Kim et al. 2003a; Soborg et al. 2002]</td>
</tr>
<tr>
<td>-allele 3 [203bp]</td>
<td>Carriage of allele 2 or 3 associated with TB in Gambians</td>
<td>[Bellamy et al. 1998b]</td>
</tr>
<tr>
<td></td>
<td>Carriage of allele 3 associated with resistance to TB</td>
<td>[Gao et al. 2000]</td>
</tr>
</tbody>
</table>

**Notation of alleles is gene-nucleotide position (allele 1/allele 2), with allele 1 being the most frequent in the population (wild-type). Amino acid changes are presented where they arise.**
TB patient may also present as prolonged, high-grade fever.

We assembled a cohort of IRD patients for studies of immunological and genetic determinants of disease. HLA-B44 and the haplotype HLA-A2, B44, TNFA-308/C3A, BAT1 (intron10)C3C, DR4 were more common in patients who experienced an IRD manifested as CMV retinitis and/or encephalomyelitis than controls [Price et al. 2001]. Patients with IRD arising from mycobacterial lymphadenitis rarely carried IL6-174*C (rs1800795, 36% versus 61/71%) and never carried TNFA-308/C3A (rs1800629, 0% versus 23/52%) [Price et al. 2002]. The study established that distinct cytokine-mediated mechanisms contribute to IRD initiated by herpesviruses and mycobacterial infections. No genetic studies of IRD associated with TB have been published. However, in a pilot study using a Cambodian cohort, TNFA-1031/C3C (rs1799964) was carried at the same frequency in HIVþ TBþ and HIVþ TB patients, so the allele did not mark susceptibility to TB in an HIV patient. However, TNFA-1031°C was slightly more common in TBþ patients who experienced an IRD compared with HIV'TBþ (p = 0.08, odds ratio [OR] = 3.41) or HIV'TBþ (p = 0.05, OR = 3.45) controls, so the allele may mark increased susceptibility to an IRD event [Affandi et al. 2009].

In conclusion, genetic studies of TB must take the ethnicity of the patients into consideration. Indeed TB (+/- HIV) is rare in Whites in the developed world. Studies of cytokine haplotypes in African and Asian populations will be critical, such as those described in the following section for TNF.

**Table 3.** Associations/dissociations of single nucleotide polymorphisms (SNPs) in immune-related genes and adult nontuberculous mycobacteria (NTM) disease.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Association with mycobacterial disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLR2 is critical for the recognition of mycobacteria by antigen presenting cells (APC)</strong></td>
<td>Minor alleles were not present in Koreans</td>
<td>Ryu et al. 2006</td>
</tr>
<tr>
<td>TLR2 Arg677Trp [C/T]</td>
<td></td>
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<tr>
<td>[rs5734706] TLR2 Arg753Gln [G/A]</td>
<td></td>
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<tr>
<td>[rs5743708]</td>
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<tr>
<td><strong>NRAMP1 encodes a divergent cation transporter which removes Fe²⁺ from phagosomes</strong></td>
<td>Heterozygosity associated with NTM lung disease (NTMLD) in Korean and Japanese cohorts</td>
<td>[Tanaka et al. 2007; Koh et al. 2005]</td>
</tr>
<tr>
<td>NRAMP1 D543N [G/A]</td>
<td></td>
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</tr>
<tr>
<td>[rs17235409] NRAMP1 INT4 [G/C]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAMP1 3'UTR [TGTG+/ deletion]</td>
<td></td>
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</tbody>
</table>

Notation of alleles is gene-nucleotide position [allele 1/allele 2], with allele 1 being the most frequent in the population (wild-type). Amino acid changes are presented where they arise.

**A review of candidate SNPs likely to affect respiratory disease**

Immune-related genes are considered in a sequence reflecting the host response to infection; specifically the recognition of the pathogen by host cells, the signals induced, the acute inflammatory response and the development of protective immunity. Associations with individual polymorphisms are summarized in Tables 1, 2 and 3.

**Pattern recognition molecules**

Eleven toll-like receptors (TLRs) have been identified in humans, with affinities for motifs characteristic of different micro-organisms. The most important here are TLR2, which interacts with lipopeptides, peptidoglycan and lipotechoid acid from the cell walls of Gram-positive bacteria, and TLR4, which interacts with the Gram-negative endotoxin, lipopolysaccharides (LPS), and pneumococcal pneumolysin [Beutler, 2002]. Homozygosity at allele 2 of TLR2-16934 (T/A) may increase the risk of Gram-positive infections including pneumonia [Sutherland et al. 2005]. Carriage of allele 2 in TLR2 Arg677Trp (C/T, rs5734706) and TLR2 Arg753Gln (G/A, rs5743708) may be associated with mycobacterial infections, TB and leprosy [Ben-Ali et al. 2004; Kang and Chae, 2001]. Carriage of allele 2 in TLR4 Asp299Gly (A/G, rs4986790) and TLR4 Thr399Ile (C/T, rs4986791) has been correlated with Gram-negative sepsis [Yuan et al. 2008; Lorenz et al. 2002]. However, other studies did not confirm these results [Everett et al. 2007; Yoon et al. 2006]. Carriage of allele 2 in TLR5 Arg392Stopcodon [1174, (C/T)], TLR5 Asn592Ser [1175, (A/G)], TLR5 1174*T-1175*A and TLR5 1174*C-1175*G were associated with an
increased risk of *Legionella pneumophila* infection [Hawn et al. 2003].

Surfactant proteins have an antigen recognition function through interaction with TLR2 and TLR4. Low levels of pulmonary surfactant proteins are associated with increased risk of pneumonia [Griese et al. 2008]. A polymorphism in surfactant protein B, SP-B *+1580 (T/C)* was associated with increased risk of pneumonia in one cohort study [Quasney et al. 2004], but no further studies have been reported.

**Signalling pathways**

Myeloid differentiation factor 88 (MyD88) adapter-like protein (MAL; also known as TIRAP) mediates downstream signalling of TLR2 and TLR4 [Beutler, 2002]. A leucine substitution at Ser180 of MAL was associated with decreased susceptibility to infectious diseases in general and specifically to pneumococcal disease [Khor et al. 2007]. Polymorphisms in *Ikappa-beta-alpha* [Chapman et al. 2007] and *Ikappa-beta-zeta* [Chapman et al. 2009] were described as risk factors for invasive pneumococcal disease by the same group. A single report of nuclear factor-kappaB (NF-kB) essential modulator (NEMO) and *IRAK-4* polymorphisms suggests these may be a risk factor for pneumococcal disease, at least in children [Ku et al. 2007].

**Pro-inflammatory cytokines**

TNF is an important pro-inflammatory cytokine and a key initial component of the inflammatory response. There is a considerable inherited variability in the TNF gene and potential for the protein to influence pneumonia and sepsis. However it lies in a highly polymorphic region of the major histocompatibility complex (MHC) on chromosome 6, making LD a major problem to be addressed.

The A allele at TNFA-308 has been associated with an increased risk of death from a variety of infectious diseases, including melioidosis [Nuntayanuwat et al. 1999]. However several studies found no association between TNFA-308 and susceptibility or outcome of CAP [Sole-Violan et al., 2009; Kinder et al. 2007; Waterer et al. 2001b]. A two-SNP haplotype including TNFA-308*A and TNF-238*A (rs361525) may be a better predictor of higher mortality in sepsis [Henckaerts et al. 2009; Pappachan et al. 2009] and at least one study found TNFA-238 allele A predicted increased risk of adverse outcome from pneumonia [Kinder et al. 2007]. Heterozygosity in the TNF soluble receptor 1B (*TNFRSF1B*+676 (Met196Arg, G/T, rs1061622)) is protective for the outcome of CAP [Sole-Violan et al. 2009].

*LTA* is adjacent to TNF on chromosome 6 and carriage of the A allele at LTA+252 (G/A, rs909253) was one of the first genetic factors associated with death from septic shock [Stuber et al. 1996]. Subsequent studies demonstrated that the patients who are homozygous for allele A in LTA+252 was also an indicator for the development of sepsis in trauma patients [Majetschak et al. 1999] and in patients with pneumonia [Waterer et al. 2001a] although other studies have not found this association [Henckaerts et al. 2009; Sole-Violan et al. 2009]. Associations with LTA+252 may reflect linked polymorphisms in the gene encoding heat shock protein-70. The candidate HSPA1B-179 (C/T, rs6457452) is in linkage with HSPA1B-1267 (A/G, rs1061581) and is associated with lower HSP-70 expression at baseline, higher TNF production after exposure to bacteria and an increased risk of septic shock. This fits current models of the pathogenesis of sepsis [Temple et al. 2004; Waterer et al. 2003].

The region spanning the TNF cluster has been implicated in susceptibility to numerous immunopathological diseases [Allcock et al. 2004], as well as CAP [Waterer et al. 2001b], but strong LD has hampered identification of polymorphisms responsible for the disease phenotypes. We investigated European, Asian and Australian Aboriginal populations to provide a framework for disease association studies using DNA from 999 unrelated healthy donors genotyped at 38 loci over a 60 kb region spanning seven genes near TNF. The PHASE algorithm was used to statistically infer TNF block haplotypes and estimate their frequencies in each population. We found just 31 haplotypes in all populations combined, with less than 19 in any single population. Four to eight TNF block haplotypes exist across all ethnicities and hence must predate the divergence of these populations from a common ancestor, possibly >160,000 years ago. Some haplotypes are unique to isolated populations but they do not contain unique SNPs [Valente et al. 2009]. Hence, they reflect restricted migration and/or extinction of some families rather than de-novo mutation. We are now examining
these haplotypes in patients with HIV-associated TB and NTM infections.

Macrophage migration inhibition factor (MIF) is released by monocytes and macrophages and exerts potent pro-inflammatory activities [Calandra and Roger, 2003]. Clinical studies associate higher circulating MIF levels with severity and susceptibility of sepsis [Gao et al. 2007]. A study of 1729 patients with CAP found 90-day mortality was lower for patients carrying the high-expression C allele of MIF-173 (G/C, rs755622) [Yende et al. 2009].

Anti-inflammatory responses

Three promoter polymorphisms in the IL10 gene [−1082 (rs1800896)/−819 (rs1800871)/−592 (rs1800872)] have been studied extensively and different −819/−592 haplotypes are associated with variable IL-10 production [Turner et al. 1997]. The effects of the IL10 haplotype may depend on whether the stimulus is Gram positive or Gram negative [Temple et al. 2003]. Several case–control studies suggest that the IL10-1082G allele is a significant risk factor for adverse outcome in patients with pneumonia [Gallagher et al. 2003; Schaaf et al. 2003]. Somewhat confusingly the IL10 haplotype −592C/734G/3367G was associated with increased mortality in patients with pulmonary sepsis, but not in similarly ill patients with extrapulmonary sepsis [Wattanathum et al. 2005]. This may reflect LD between IL10 and FCGR2 polymorphisms, as discussed earlier.

Pathways activated by inflammation

Activation of the coagulation system may influence multiorgan dysfunction in sepsis [Bastarache et al. 2006]. For example, mice heterozygous for factor V Leiden status have improved survival after exposure to LPS [Weiler et al. 2004] and the efficacy of activated protein C was greater in sepsis patients carrying the factor V Leiden mutation [Yan and Nelson, 2004]. A prospective cohort study in 3347 patients with pneumonia associated a promoter haplotype (Plasminogen activator inhibitor-1, PAI 4G/2846G/4588C/7343A) with increased susceptibility to pneumonia, with PAI-1 levels after stimulation 20% higher in subjects with a 4G genotype as compared with those with 5G [Yende et al. 2007]. A further study found an association with mortality and carriage of the 4G allele of PAI-1 [Sapru et al. 2009], but other polymorphisms were not assessed so it is not clear whether this reflects the same haplotype.

Understanding genetic associations: Caveat emptor

Genetic studies have appeal to clinical researchers because the time of sample collection is flexible, DNA samples are robust and the assays are easily automated. Finding a sensitive and specific test of susceptibility or a novel pathogenic pathway has appeal. However, many SNPs do not associate with disease, so it is important to emphasize that we conclude nothing from null associations. A positive association places the gene (or one in LD) somewhere in the path to disease, but LD precludes assumptions about role of the SNP. A null association will occur if the SNP (and its haplotype) do not affect gene expression, or if expression is not limiting in the disease process (so it does not matter if it varies). The reports reviewed herein show that effective genetic studies require detailed interpretation of the associated haplotypes with consideration to ethnic background and confounding factors.

Acknowledgments

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Conflict of interest statement

None declared.

References


positions/C0 transcription following stimulation of peripheral blood cells with Streptococcus pneumoniae. *Immunogenetics* 55: 629–632.


ERRATUM

In the process of putting this thesis together, a minor error was noticed that occurred through three publications.

In Chapters 3, 4 and 5:
The TNFA-1031 was incorrectly identified the major allele being C and the minor allele as T. The correct presentation of TNFA-1031 should be major allele as T and minor allele as C.

As I am presenting these chapters as the published research articles, no changes have been made as the data and analysis are not affected and still remain correct.

In Chapter 4:
Homozygous carriage of the variant allele in IL1A+4845 was significantly associated with increased risk of developing non-tuberculous mycobacterial (NTM) disease. This was not addressed in chapter 4 when it was a significant finding. Significance of this SNP has been addressed in Section 1.7 of Chapter 7 – Discussion.
Chapter 3

The search for a genetic factor associating with immune restoration disease in HIV patients co-infected with Mycobacterium tuberculosis.


This chapter is presented in the form accepted for publication and constitutes pages 34-38 of this thesis.

*Chapter outline:*

This chapter focuses on identifying genetic polymorphisms in cytokine, chemokine and their receptors, associated with susceptibility to an increased risk of developing an IRD event in HIV patients co-infected with TB from two sites – Cambodia and India.
Brief Communication

The search for a genetic factor associating with immune restoration disease in HIV patients co-infected with *Mycobacterium tuberculosis*

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\(^b\)Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India
\(^c\)Lala Ram Sarup Institute of Tuberculosis and Respiratory Diseases, New Delhi, India

Abstract.

**BACKGROUND:** Up to 43% of HIV-infected patients co-infected with *Mycobacterium tuberculosis* experience exacerbations of tuberculosis (TB) after commencing antiretroviral therapy (ART). These are termed immune restoration disease (IRD). It is unclear why individual susceptibility varies.

**OBJECTIVE:** We investigate if single nucleotide polymorphisms (SNP) in genes encoding cytokines, chemokines and their receptors associate with development of an IRD event in patients of two different ethnicities.

**METHODS:** DNA samples were available from small well-characterised groups of HIV patients treated in Cambodia (TB-IRD, \(n = 17\); HIV\(^+\)TB\(^+\) controls, \(n = 55\)) and India (TB-IRD, \(n = 19\); HIV\(^+\)TB\(^+\) controls, \(n = 43\)). HIV patients with a TB diagnosis but no evidence of IRD were included to control for susceptibility to TB per se. Sixteen SNP implicated in inflammation or mycobacterial disease were genotyped.

**RESULTS:** Susceptibility to TB-IRD associated with carriage of TNFA-1031*T (rs1799964; \(P = 0.05\)) and SLC11A1 D543N*G (rs17235409; \(P = 0.04\)) in Cambodian patients and carriage of IL18-607*G (rs1946518; \(P = 0.02\)) and VDR FokI (F/F)*T (rs10735810; \(P = 0.05\)) in Indian patients.

**CONCLUSIONS:** Associations between polymorphisms in immune-related genes and TB-IRD were found, but none were common across two ethnicities.

Keywords: TB-IRD susceptibility, immunogenetic factor, single nucleotide polymorphism

1. Introduction

Tuberculosis is the most common opportunistic infection associated with HIV and is the leading cause of morbidity and mortality in people living with HIV/AIDS in resource-limited settings. Combination antiretroviral therapy (ART) has reduced the incidence and severity of *Mycobacterium tuberculosis* (TB) infections in HIV patients. When they begin ART, a proportion of HIV-TB co-infected patients experience atypical presentations of tuberculosis termed immune restoration disease (TB-IRD). This can occur despite favourable virological responses and increases in CD4\(^+\) T-cell counts on ART [8,10]. TB-IRD is com-
common in developing countries where TB is endemic such as Cambodia and India [2,4]. We have sought common disease-associated polymorphisms by investigating genetic associations in patients of different ethnicities from the developing world. Samples were available from HIV patients from Cambodia and India who presented with TB-IRD, as well as HIV-positive TB-positive control patients.

Single nucleotide polymorphisms (SNP) affecting cytokines, chemokines and their receptors may influence development of an IRD event via effects on HIV disease, on TB itself or on the response to ART [10]. We evaluated single nucleotide polymorphisms (SNP) in inflammatory and other immune-related genes selected based on a literature search for laboratory evidence of functional consequences for an allele and associations with autoimmune disorders, infectious diseases, respiratory and mycobacterial diseases [1]. Here we present the associations of immune-related genotypes on the risk of developing IRD in TB patients. A significant association places this SNP or one in linkage disequilibrium in the path to disease. If the association is similar in two ethnicities, one can argue that the SNP is biologically important and where differences are observed between ethnicities, one can argue that the effect is random or that the haplotypic associations around the SNP vary with ethnicity.

2. Methods and materials

DNA samples were available from two prospective cohort studies of HIV patients beginning ART with known TB status. Patients were monitored for 6–12 months to establish the occurrence of IRD using guidelines by the treating clinicians as proposed by the International Network for the Study of HIV-associated IRIS [8]. Both studies were approved by Human Research Ethics Committees of the relevant institutions and all participants provided written informed consent.

1. HIV+ TB+ patients were recruited from the National Centre for HIV/AIDS, Dermatology, and Sexually Transmitted Disease, Social Health Clinic, in Phnom Penh, Cambodia [4]. These included 10 patients with paradoxical TB-IRD (ie: whilst on TB treatment) and 7 with ART-associated TB-IRD (ie: a new diagnosis of TB on ART) [total TB-IRD patients (n = 17) and HIV+ TB+ controls (n = 55)].

2. TB-IRD patients (n = 19; all paradoxical TB-IRD) and HIV+TB+ controls (n = 43) were recruited at the Lala Ram Sarup (LRS) TB and Respiratory Diseases (Delhi, India) after initiating TB therapy [2,13].

DNA samples were extracted from PBMC or saliva using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) and stored at −80°C. Sixteen SNPs were genotyped using TaqMan FAM or VIC-labelled probes and Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). BAX intron 10 (rs9281523) was genotyped using a custom TaqMan assay [17]. DNA samples were diluted to 10 ng/µL and amplified on an ABI Prism 7900 HT (Applied Biosystems) or Roche LC480 (Roche Applied Science, Indianapolis, IL, USA) in 384-well microtiter plates. Thermocycling involved one step of 10 min at 95°C, followed by 40 cycles of denaturation at 92°C for 15 s with annealing and extension at 60°C for 1 min. Fluorescence was read on an ABI 7900 Sequence Detector (Applied Biosystems) or Roche LC480 (Roche Applied Science) using proprietary allelic discrimination software. Repeats and non-template controls were included in each run. The median (range) genotyping success rate was 99% (98–100%) for each SNP in both cohorts. Alleles are recorded as “gene-nucleotide position*allele 1/allele 2”, with allele 1 being the most frequent allele in Caucasians (previously defined as wildtype). In accordance with our earlier publications [15,16], TNF polymorphisms are described by their “traditional” names, TNF-308 and TNF-1031. We note that they lie 307 and 1030 bases (resp.) from the transcription start site [14].

Allele and genotype frequencies in each ethnic group conformed with Hardy-Weinberg equilibrium (HWE) when assessed using GENEPOP v3.3 [11]. Fisher’s exact test was used to identify factors associated with risk of developing TB-IRD. Results are presented as Odds ratios (OR), their 95% confidence intervals (95% CI) and P values, using the homozygous wild-type allele as the reference group. P ≤ 0.05 was accepted as indicating a significant difference. Analyses were performed using GraphPad (Prism version 5.02 for Windows, GraphPad Software, San Diego, CA, USA). Logistic regression analyses utilised Stata (StataCorp. 2009, Stata Statistical Software: Release 11. College Station, Texas, USA).

3. Results

A logistic regression that included all SNP, both cohorts and CD4T-cell counts (square root transformed
to approximate a normal distribution) generated significant associations with heterozygosity at IL1A + 4845 (p = 0.05) and IL18-607 (p = 0.014). Sequential removal of SNP that were not associated with TB-IRD yielded a significant fit (p = 0.0175) with a model including heterozygosity at SLC1A1 D543N (p = 0.036) and IL18-607 (p = 0.026). However these analyses do not reveal ethnic differences, so we present simple bivariate associations (Table 1).

In Cambodian patients, carriage of allele T in TNFA-1031 (rs1799964) and allele G in SLC1A1 D543N (rs17235409) [P = 0.05, OR = 3.6; P = 0.04, OR = 0.21; resp] was associated with susceptibility to TB-IRD. Trends were observed with carriage of allele A in IL1A + 4845 and allele T in IL2-330 (P = 0.08 and P = 0.09 resp). In Indian TB patients, carriage of allele G in IL18-607 and allele T in VDR FokI (F/f) was associated with increased incidence of susceptibility to TB-IRD (P = 0.02, OR = 3.8; P = 0.05, OR = 3.3, resp).

4. Discussion

The results show distinct patterns of association in the two populations. For example; carriage of the variant allele in SLC1A1 D543N conferred protection from an IRD event in Cambodians but there was no effect in Indian patients. This SNP has been associated with susceptibility to inflammatory diseases such as Crohn’s disease in Greek patients [5] and Buruli ulcers in Ghanaian patients [12]. Carriage of the variant allele in IL18-607 conferred an almost 4-fold increased risk of developing an IRD event in Indian patients, but had no effect in Cambodians. No such association was observed in a cohort of HIV-negative Indian pulmonary TB patients [7], but in a Han Chinese pulmonary TB cohort without HIV, carriage of the variant allele in IL18-607 was associated with a decreased risk of developing TB [6].

Ethnic differences may arise when the SNP assayed is not directly responsible for the effect, but lies in a haplotype that is differentially carried in donors of each ethnicity. This cannot be determined for IL18 and SLC1A1 at this time, but there is more information about TNF. Carriage of the variant allele in TNFA-1031 (rs1799964) in TB-IRD patients associated with a 4-fold greater risk in Cambodians, but this was not evident in the Indian cohort. TNFA-1031*2 affects neuropathy in Malay and Chinese HIV-infected patients [3] and haplotype blocks carrying TNF-1031 differ in frequency between Indian, Chinese and Cambodian patients [3] and haplotype blocks carrying TNF-1031 differ in frequency between Indian, Chinese and Cambodian patients [3].
The haplotype incorporating allele 2 at TNFA-308 and BAT1(int10) is present in Asians [16], so there is a clear dissociation between the role of TNF haplotypes in IRD associated with TB in the developing world (Table 1) and non-tuberculous mycobacteria in Australia [9].

Overall there were associations between polymorphisms in immune-related genes and TB-IRD, but none held in both ethnicities. Although the cohorts were small, it can be argued that effects strong enough to have a use in clinical practise should have emerged. Subsequent studies should investigate patterns of linkage disequilibrium to determine whether the associations observed here reflect ethnic differences in the haplotypic structures across critical genes.

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Conflict of interest

The authors have no conflicts of interest to declare.

References


Chapter 3a

Short Communication: Plasma Levels of Vitamin D in HIV Patients Initiating Antiretroviral Therapy Do Not Predict Immune Restoration Disease Associated with Mycobacterium tuberculosis


This chapter is presented in the form accepted for publication and constitutes pages 39-42 of this thesis.

NOTE: Journal article removed due to copyright restrictions.

Chapter outline:

This appendix focuses on investigating whether plasma vitamin D levels and polymorphism in the vitamin D receptor can predict an IRD event in HIV patients co-infected with TB.
Chapter 4

Searching for an immunogenetic factor that will illuminate susceptibility to non-tuberculous mycobacterial disease


This chapter is presented in the form accepted for publication and constitutes pages 39-42 of this thesis.

*Chapter outline:*

This chapter focuses on investigating the potential of polymorphisms affecting cytokine, chemokine and their receptors, influencing the development of NTM disease.
Rapid Communication

Searching for an immunogenetic factor that will illuminate susceptibility to non-tuberculous mycobacterial disease

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ABSTRACT

The incidence of pulmonary non-tuberculous mycobacteria (NTM) disease in otherwise healthy adults is increasing as the population ages. The organisms are ubiquitous so susceptibility probably reflects a deficiency in a protective immune response. Here we investigate if single nucleotide polymorphisms (SNP) affecting cytokines, chemokines and their receptors associate with pulmonary NTM disease. Samples from NTM patients (n = 79) and healthy controls (n = 188) were genotyped using TaqMan probes. Of the 16 SNPs assessed, IL28B-rs8099917TG (rs8099917; p = 0.01, OR = 2.2), TNFA-1031′CC (rs1799964; p = 0.02, OR = 0.48) and IL10-1082′AA (rs1800896; P = 0.001, OR = 0.33) were significantly associated with NTM disease. IL28B-rs8099917 and IL10-1082 have been associated with perturbations of the Th1/Th2 balance, whilst TNFA-1031′CC associates with sensory neuropathy in HIV patients. IL10-1082 warrants further investigation because we observed high production of IL-10 in blood mononuclear cells from NTM patients.

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1. Introduction

The prevalence of pulmonary non-tuberculous mycobacteria (NTM) disease is rising worldwide [1–6]. Most cases involve Mycobacterium avium and/or intracellulare [7], which are common in the environment and grow in biofilms lining suburban drinking water pipes [8]. NTM disease is often indistinguishable clinically and radiographically from diseases caused by Mycobacterium tuberculosis, but most patients have no identifiable risk factor. NTM disease is most common in people over 50 years of age, so an increasing disease burden is inevitable as the population ages [2,9]. The available treatments are expensive and poorly tolerated, and reinfection or relapses are common. Clearance of mycobacteria requires an effective Th1 cell-mediated immune response [10] mediated by interferon (IFN)-γ and driven by IL-12 and tumour necrosis factor (TNF)-α [11]. Th1 responses can be inhibited by Th2 cytokines and regulatory T-cells (Treg). IL-12 induces production IFN-γ and the activation, differentiation, and expansion of antigen-specific Th1 cells [12]. As IL-23 is required to induce Th17 responses, combined IL-12 and IL-23 deficiency due to a mutation in the common p40 subunit (encoded by IL12B) is associated with a more severe immunodeficiency than IL-12 deficiency alone [13].

Single nucleotide polymorphisms (SNP) affecting cytokines, chemokines and their receptors may influence development of NTM disease. We evaluated sixteen SNPs in inflammatory and other immune-related genes selected based on a literature search using laboratory evidence suggesting functional consequences for an allele and associations with autoimmune disorders or infectious diseases [14]. A significant association places this SNP or one in linkage disequilibrium with it in the path to disease.

2. Methods and materials

DNA samples were available from Australian (Caucasian) patients with pulmonary NTM disease (n = 79) attending Royal Perth Hospital (Western Australia), Prince Charles Hospital and Greenslopes Hospital (Queensland). All patients had been diagnosed using standard guidelines of the American Thoracic Society [15], had disease due to M. intracellulare or M. avium and a median (range) age of 72 (41–97) years and a male to female ratio of 50:29. In short, they have progressive radiologically active disease and repeated culture from sputum of NTM of culture from a bronchoalveolar lavage or lung biopsy specimen. In our region, more than 95% of pul-

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and stored at NTM disease is associated with alleles of TNFA-1031 and IL10-1082 using a son, with a median (range) age of 51 (18–87) years and a male to hort from the Busselton Survey (n = 188) were used as a compari-

sion, with a median (range) age of 51 (18–87) years and a male to female ratio of 96:102. This study was approved by the Ethics Review Boards of participating institutions, and informed consent was given by the individuals and/or their guardians.

DNA samples were extracted from PBMC or saliva samples using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) and stored at −80 °C. Sixteen SNPs were genotyped using custom TaqMan FAM or VIC-labelled probes and Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) (See Table 1). rs9281523 [16] and

rs12979860 [17] were genotyped using custom TaqMan assays. DNA samples were diluted to 10 ng/μL and amplified on an ABI Prism 7900 HT (Applied Biosystems) or Roche LC480 (Roche Applied Science, Indianapolis, IN, USA) in 384-well microtiter plates. Thermocycling involved one step of 10 min at 95 °C, followed by 40 cycles of denaturation at 92 °C for 15 s with annealing and extension at 60 °C for 1 min. Fluorescence was read on an ABI 7900 Sequence Detector (Applied Biosystems) or Roche LC480 (Roche Applied Science) using proprietary allele discrimination software. Repeats and non-template controls were included in each run. The median (range) genotyping success rate was 99% (98–100%) for each SNP.

Allele and genotype frequencies in each ethnic group conformed with Hardy–Weinberg equilibrium (HWE) when assessed using GENEPOP v3.3 [18]. Logistic regression modelling was used to identify factors independently associated with risk of developing NTM disease. All factors associated with NTM disease in univariate analysis were included in a multivariable model and a manual backward step-wise removal procedure was used to obtain the final model for each outcome. Results are presented as Odds Ratios (OR), their 95% confidence intervals (95% CI) and P values, using the homoygous wild-type allele as the reference group. P < 0.05 was accepted as indicating a significant difference. Analyses were performed using Stata (StataCorp. 2009, Stata Statistical Software: Release 11, College Station, Texas, USA) as indicated in the tables.

3. Results and discussion

Univariate analyses presented in Table 1 show significant associations with TNFA-1031 (C/T, rs1799964) and IL10-1082 (A/G,
rs1800896) and weak associations with alleles of IL1A and IL28. Multivariable analyses derived by stepwise removal of the weakest associations are presented in Table 2. IL1A alleles were not associated with NTM disease in the multivariable analysis.

Carriage of the IL28B-rs8099917'TG was marginally more common in patients than controls and carriage of the G allele emerged as a significant difference in the multivariate analysis (Univariate: P = 0.06, OR = 1.76, see Table 1; Multivariate: P = 0.01, OR = 2.2, see Table 2). This associates IL28B-rs8099917'TG with susceptibility to NTM disease. Carriage of the G allele at IL28B-rs8099917 predicts a poor outcome following interferon (IFN)-based treatment of Hepatitis C virus (HCV) infection [19–21]. This may reflect the antiviral or immunomodulatory properties of IL-28 (IFN-λ).

Carriage of the heterozygous CT allele at TNFA-1031 had 52% lower odds of developing NTM disease when compared to patients carrying TNFA-1031‘CC (Univariate: P = 0.03, OR = 0.50, see Table 1; Multivariate: P = 0.03, OR = 0.48, see Table 2). This associates carriage of TNFA-1031‘CC with susceptibility to NTM disease. TNFA-1031‘T has been associated with sensory neuropathy in HIV patients exposed to stavudine [22]. It is notable here that neither TNFA-308‘A nor BAT1 intron 10‘C is associated with NTM disease. These alleles mark the Caucasian HLA-A1,B8,D83 haplotype that has multiple disease associations [23].

IL10-1082‘AG was less common in patients with NTM disease than in controls. The data suggests that the IL10-1082‘AG genotype carries 67% decreased odds of developing NTM disease compared to carriage of the IL10-1082‘AA genotype (Univariate: P = 0.004, OR = 0.40; Multivariate: P = 0.01, OR = 0.33). This suggests that carriage of IL10-1082‘AA confers susceptibility to NTM disease. This SNP has diverse disease associations and its effect on transcription is unclear. IL10-1082‘AA associated with increased serum IL10 levels in New Zealand patients with Crohn’s disease [24] and the IL10-1082‘A allele conferred a twofold increase in transcriptional activity of the IL10 promoter compared to the G allele [25]. This fits evidence from our cohort that NTM patients exhibit high production of IL-10 by blood mononuclear cells cultured with mycobacterial extracts [26]. However in other studies, carriage of IL10-1082‘A associated with lower levels of IL10 production [27] and homozygous carriage of IL10-1082‘GG was associated with higher circulating IL-10 [27], higher expression of IL10 mRNA [28] and greater production of IL10 after in vitro stimulation [29].

We considered the possibility that the association between IL10-1082‘AA and susceptibility to NTM disease may be an artefact created by the exclusion of comorbidities from the patient cohort whilst the control cohort was younger may later develop NTM disease and/or comorbidities. We assessed a cohort of healthy older donors (n = 181) with a median (range) age of 68 (55–82) years recruited as controls for a study of venous leg ulcers. Candidates were excluded if they had overt infectious disease, abnormal venous function, type 1 diabetes or rheumatoid arthritis [16]. The distribution of IL10-1082 alleles was AA:32, GA:103, GG:41 (vs AA:38, GA:109, GG:41 for the controls described in Table 1, P = 0.69, OR = 0.88, 95%CI = 0.5–1.5; Fisher’s Test). Hence carriage of the G allele in IL10-1082 still conferred protection against NTM disease (P = 0.001, OR = 0.37, CI = 0.2–0.67; Fishers’ test) when this older control cohort was used.

SLC11A1 D543N has been associated with M. avium complex (MAC) disease in Asians [30,31]. Our results did not achieve significance but this may reflect the difference in ethnicities.

Overall there were associations between polymorphisms in immune-related genes and NTM. Subsequent studies should investigate patterns of linkage disequilibrium in larger cohorts to determine whether the associations observed here are random or reflect ethnic differences in the haplotype structures across critical genes.

**Conflict of interest**

The authors have no conflicts of interest to declare.

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The authors thank all patients and controls who donated samples for this study.

**References**


Can immune-related genotypes illuminate the immunopathogenesis of cytomegalovirus disease in human immunodeficiency virus-infected patients?


This chapter is presented in the form accepted for publication and constitutes pages 47-57 of this thesis.

*Chapter outline:*

This chapter focuses on investigating immune-related genetic polymorphisms and their association with susceptibility to increased risk of developing CMV end organ disease and predisposition to low nadir CD4 T cell counts.
Can immune-related genotypes illuminate the immunopathogenesis of cytomegalovirus disease in human immunodeficiency virus–infected patients?

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Abstract

Most human immunodeficiency virus (HIV) patients are seropositive for cytomegalovirus (CMV) but a smaller proportion experience end-organ disease. This observation may reflect variations in genes affecting inflammatory and natural killer cell responses. DNA samples were collected from 240 HIV-infected patients followed at the University Hospitals/Case Medical Center (Cleveland, OH) between 1993 and 2008. Seventy-eight patients (African Americans = 41, Caucasians = 37) experienced CMV disease. Genotypes were determined using allele-specific fluorescent probes or multiplex polymerase chain reaction sequence-specific primers. IL12B3 UTR*(1) and SLCl1A1 D543N*(1,2) were associated with CMV disease in African American patients (p = 0.04 and p = 0.02, respectively). IL10-1082*(1,2) and LILRB1 I142T*(1) were associated with CMV disease in Caucasians (p = 0.02 and p = 0.07, respectively). DARC T-46C*(1) and C14C-159T*(2) were associated with low nadir CD4+ T cell counts in African American patients (p = 0.02 and p = 0.01, respectively). Caucasian patients carrying TNFA-308*2, TNFA-1031*(2), IL2-330*(1), CCL2-2518*(2), or LILRB1 I142T*(1) had significantly lower nadir CD4+ T cell counts in a bootstrapped multivariable model (p = 0.006–0.02). In general, polymorphisms associated with CMV disease and CD4+ T cell counts were distinct in Caucasian and African American patients in the United States. The LILRB1 I142T polymorphism was associated with both CMV disease and low nadir CD4+ T cell counts in Caucasians, but the clearest determinant of low nadir CD4+ T cell count in African American patients was DARC T-46C.

1. Introduction

Cytomegalovirus (CMV) is a herpesvirus that often causes asymptomatic infections in healthy individuals, but can cause more serious disease in the immune compromised population. In persons with acquired immune deficiency syndrome (AIDS), retinitis was a common complication of CMV infection [1,2]. In patients initiating antiretroviral therapy, CMV may result in immune restoration disease (IRD) presenting as retinitis or uveitis. CMV IRD is most common among HIV patients who begin treatment with CD4+ T cell counts below 100 cells/μL [3].

Most HIV+ patients are seropositive for CMV [4], but only a subset develop CMV disease. Innate and adaptive immune responses that affect susceptibility to CMV can be identified by the identification of genetic polymorphisms that associate with the occurrence of CMV end-organ disease. Polymorphisms in immune-related genes may impact the recurrence or re-emergence of CMV in HIV patients if they favor T cell activation, apoptosis, or exhaustion, promoting extremely low nadir CD4+ T cell counts. In Australian Caucasian HIV+ patients, CMV disease has been associated with the 44.1 ancestral haplotype (HLA-A2, B44, TNFA-308*2, BAT1(intron10)*1, DR3) and carriage of IL12B3 UTR*(1) (rs3212227, A/C) [5].

Natural killer (NK) cells play a fundamental role in controlling viral infections such as CMV. The absence of functional NK cells may influence susceptibility to CMV disease [6]. NK cell function and numbers are reduced in HIV-infected patients as the disease progresses [7]. NK cells express activating and inhibitory receptors capable of recognizing host cells using classical and nonclassical major histocompatibility complex class I molecules (MHC-I). Of these, the leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1 encoded by the LILRB1 gene) is an inhibitory receptor that recognizes MHC-I molecules, including human leukocyte antigen (HLA)-G. Killer immunoglobulin-like receptors (KIR) interact with HLA-B, HLA-C, or HLA-G on targeted cells and
also regulate NK cell activity [8]. There are 16 known KIR genes encoding both inhibitory and activating receptors [9], which can affect the outcome of diseases such as HIV-1 infection and CMV end-organ disease [10]. Patients who experienced CMV disease as an AIDS-associated illness or an IRD had more activating KIR than did matched controls [11]. HLA-G is a nonclassical MHC-I molecule that may regulate the function of NK cells, T cells, and dendritic cells. The inhibitory functions of HLA-G may be exerted upon interaction with LILRB1, LIR-2, or KIR2DL4. Uptregulation of these molecules on NK cell lines has been associated with overexpression of HLA-G, which may facilitate immune evasion [12]. Homozygous carriage of the 14-base pair (bp) deletion polymorphism in HLA-G has been associated with susceptibility to CMV disease [12].

Racial differences influence the incidence and outcome of inflammatory disorders such as sepsis [13]. People of African descent may have genotypes that favor a “high expression profile” of pro-inflammatory cytokines and a “low expression profile” of anti-inflammatory cytokines compared with people of European descent [14,15]. Self-identified race and ethnicity have been accepted as categorization for genetic and epidemiologic studies [16]. In the current study we used cohorts of distinct ethnicity but from the same clinic to investigate whether immunoregulatory, immune-related genotypes and/or polymorphisms affecting NK cells can render a patient more susceptible to CMV disease.

This study aims to establish the genetic basis for an HIV patient’s susceptibility to CMV end-organ disease. We evaluated 16 single nucleotide polymorphisms (SNPs) in inflammatory and other immune-related genes, the 14-bp indel in HLA-G, and carriage of the KIR genes (listed in Table 1). Variants were selected based on a literature search using laboratory evidence suggesting functional consequences for an allele and associations with autoimmune disorders or infectious diseases. Because CMV is a disease of extreme immunodeficiency, genetic mechanisms that drive CD4+ T cell depletion or affect survival when counts become low may underlie genetic associations with CMV disease. Here we present results for the risk of (a) developing CMV end-organ disease and (b) low nadir CD4+ T cell counts.

2. Subjects and methods

2.1. Study cohort

Peripheral blood mononuclear cells (PBMC) and nadir CD4+ T cell counts (the lowest count recorded) were collected from patients between 21 and 61 years old attending the Special Immunology Unit of University Hospital/Case Medical Center, Cleveland, between the years 1993 and 2008. Cases were defined as HIV-infected patients (African Americans = 41, Caucasians = 37) with a history of CMV end-organ disease. Controls were defined as CMV-seropositive patients with no history of CMV end-organ disease (African Americans = 114, Caucasians = 48). The study was approved by the human subjects institutional review boards of the participating institutions, and informed consent was given by the individuals and/or their guardians.

2.2. Genotyping

DNA was extracted from PBMC using a QiAamp DNA blood mini kit (Qiagen, Valencia, CA) and stored at −80°C. Sixteen SNP were genotyped using TaqMan FAM or VIC-labeled probes (see Table 1) and Universal PCR Master Mix (Applied Biosystems, Foster City, CA), rs9281523 was genotyped using a custom TaqMan assay [17]. DNA samples were diluted to 10 ng/μL and amplified on an ABI Prism 7900HT in 384-well microtiter plates (Applied Biosystems, Foster City, CA). Thermal cycling involved 1 step of 10 minutes at 95°C, 40 cycles of 92°C for 15 seconds with annealing, and extension at 60°C for 1 minute. Fluorescence was read on an ABI 7900 sequence detector (Applied Biosystems) and Roche Lightcycler® 480 real-time PCR system (LC480) (Roche Applied Science, Indianapolis, IL) using proprietary allelic discrimination software. Repeats and nontemplate controls were included in each run. Median (range) genotyping success rates were 99% (98–100%) in all cohorts. Alleles are recorded as “gene-nucleotide position”/allele 1/allele 2,” with allele 1 being the most frequent allele in Caucasians (wild type).

KIR typing was possible in a subset of African American patients (cases, n = 31; controls, n = 41) based on a multiplex polymerase chain reaction (PCR) sequence-specific primer method using 27 locus-specific primers in 4 pools [18]. PCR reactions were performed in 10-μL volumes containing 1 μL 10× PCR buffer (Invitrogen, Carlsbad, CA), 1 μL 10 mM dNTP (Invitrogen), 0.9 μL 25 mM MgCl2 (Invitrogen), 4 μL forward and reverse primers, 15 ng template DNA, and 0.16 μL 250 U HotStar Taq DNA polymerase (Qiagen, GmbH, Hilden, Germany) amplified by denaturation (93°C, 15 minutes) followed by 32 cycles of 93°C for 20 seconds, 65°C for 30 seconds, and primer extension at 72°C for 30 seconds. PCR products were screened by 3% agarose gel electrophoresis (150 V, 30 minutes).

The HLA-G C→T 14-bp indel polymorphism was typed using primers 5′-GTG ATG GCC TGT TTA AAG TGT CAC C–3′ and 5′-GGAGAATGACTTCTAGCATGA–3′. PCR reactions were performed in 20-μL volumes containing 2 μL 10× PCR buffer containing 20 mM MgCl2, 1 μL 40 mM dNTP mix, 1 μL primers, 15 ng template DNA, and 0.3 μL 5 U Platinum Taq DNA polymerase amplified using 95°C for 55 minutes and then 35 cycles of 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds [19]. PCR products were screened by 3% agarose gel electrophoresis (150 V, 30 minutes).

2.3. Statistical analyses

Allele and genotype frequencies in each ethnic group conformed with Hardy–Weinberg equilibrium when assessed using GENEPOP v. 3.3 (Laboratoire de Genetique et Environment, Montpellier, France) [20].

Logistic regression modeling was used to identify factors independently associated with the risk of developing CMV disease. Exact logistic regression was used when numbers in genotype categories were small. Associations between nadir CD4+ T cell counts and carriage of variant alleles were investigated using negative binomial regression. All factors associated with the outcomes in univariate analysis were included in a multivariable model and a manual backward stepwise removal procedure was used to obtain the final model for each outcome and ethnicity. The resulting multivariable model was then bootstrapped to avoid overfitting. Results are presented as odds ratios (OR) or incident rate ratios (IRR), their 95% confidence intervals (95% CI), and p values using the homozygous wild-type allele as the reference group. Summary statistics including the median and interquartile range (IQR, 25–75%) for CD4+ T cell counts and proportions with CMV disease are also provided. Associations between KIR genotypes and CMV disease or nadir T cell counts were assessed using 2-sided Fisher’s exact tests and unpaired Student t tests. p < 0.05 was accepted as indicating a significant difference. Analyses were performed using GraphPad (Prism v. 5.02 for Windows; GraphPad Software, San Diego, CA) or Stata (StataCorp 2009 Stata Statistical Software, release 11, College Station, TX) where indicated in Tables 2 and 3, and Supplementary Tables 1 and 2.

3. Results

3.1. Allelic frequencies of African Americans and Caucasians differ

Several allelic frequencies differed between African Americans and Caucasians; specifically, IL12B 3’UTR (p = 0.0004, OR = 2.7, 95% CI = 1.6–4.8), IL1A+4845 (p = 0.002, OR = 0.4, 95% CI = 0.25–0.7), IL4-589 (p = 0.0001, OR = 18.5, 95% CI = 9.3–37), CD14*159 (p = 0.0005, OR = 0.35, 95% CI = 0.2–0.6), IL2-330 (p = 0.0001, OR = 0.2,
Table 1
List of genes and polymorphisms

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP (common name)</th>
<th>WT/variant</th>
<th>rs No.</th>
<th>TaqMan catalog No.</th>
<th>Effect of the minor allele</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Inflammation and immune-related SNPs</strong></td>
<td></td>
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<tr>
<td>TNFA</td>
<td></td>
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<tr>
<td>Tumor necrosis factor-α</td>
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<tr>
<td>TNFA-308</td>
<td>G/A</td>
<td>1,800,629</td>
<td>C_7514879_10</td>
<td>Elevated levels in serum</td>
<td>Inflammatory bowel disease</td>
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<td>C/T</td>
<td>1,799,964</td>
<td>C_7514871_10</td>
<td>Antiretroviral toxic</td>
<td>Neuropathy</td>
<td>[46]</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>BAT1 (intron10)</td>
<td>G/A</td>
<td>9,281,523</td>
<td>C_7514873_10</td>
<td>Increased secretion of IL1α</td>
<td>RA, poor virological response in HIV patients</td>
<td>[48–50]</td>
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<td>C_9546471_10</td>
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<td>RA</td>
<td>RA</td>
<td>[52,53]</td>
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<td>C_16176216_10</td>
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<tr>
<td>IL10-1082</td>
<td>A/G</td>
<td>1800896</td>
<td>C_1747360_10</td>
<td>Decreased IL-10 expression</td>
<td>RA</td>
<td>[55]</td>
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<td>[56]</td>
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<td>[57]</td>
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<td>RA</td>
<td>[58]</td>
</tr>
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<tr>
<td>Chemokine (C–C motif)</td>
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<td></td>
<td></td>
<td></td>
<td>Atherosclerosis in HIV patients</td>
<td>[59]</td>
</tr>
<tr>
<td>ligand 2</td>
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<td></td>
<td>Atherosclerosis in HIV patients</td>
<td>[59]</td>
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<td>[60]</td>
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<td>Duffy antigen receptor</td>
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<td>[61]</td>
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<tr>
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<td>member 1 SLC11A1 D543N</td>
<td>G/A</td>
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<td>C_25635296_10</td>
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<td>Cluster of differentiation</td>
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<td>14 CD14 C-159T</td>
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<td>C_16043997_10</td>
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<td>RA</td>
<td>[64]</td>
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<td><strong>Polymorphisms and genes associated with natural killer cell activity</strong></td>
<td></td>
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<td><strong>LIR</strong></td>
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<tr>
<td>Leukocyte immunoglobulin-</td>
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<tr>
<td>like receptor 1 LILRB1</td>
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<td>RA</td>
<td>[65]</td>
</tr>
<tr>
<td><strong>HLAG</strong></td>
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<td></td>
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<tr>
<td>HLA-G*</td>
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<tr>
<td><strong>KIR</strong></td>
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<td></td>
</tr>
<tr>
<td>KIR</td>
<td></td>
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</tr>
</tbody>
</table>

AIDS, acquired immune deficiency syndrome; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor; NK, natural killer; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; WT, wild type.

3.2. Associations with CMV disease

In African American patients, the odds of developing end-organ CMV disease in patients heterozygous at IL12B3 UTR were 55% lower than for patients homozygous for the wild-type genotype (p = 0.04, OR = 0.45, 95% CI: 0.21–0.98; as indicated in Table 2). By contrast, heterozygosity at SLC11A1 D543N increased the risk of CMV disease (p = 0.02, OR = 3.3, 95% CI = 1.2–8.9; as indicated in Table 2). No significant differences were observed with the remaining polymorphisms (listed in Suppl Table 1).

CMV disease was not associated with alleles of the LILRB1 1142T or HLA-G*14–14-bp indel in African American patients (p = 0.65 and p = 0.55, respectively, as indicated in Table 2 and Suppl Table 1). 3DS1, an activating KIR gene, appeared more common in patients with no history of CMV disease, but this did not reach significance (p = 0.21, OR = 0.38, CI = 0.09–1.5; Fisher’s exact test). No other activating and inhibitory KIR genes were associated with risk of CMV disease (Student’s t test, p = 0.58–1.00; as indicated in Table 3).
In Caucasian patients, heterozygosity at IL10-1082 was associated with higher odds of developing CMV disease (p = 0.02, OR = 3.5; 95% CI = 1.2–10; as indicated in Table 2). Heterozygosity at LILRB1 I142T (T/C) was associated with decreased odds of CMV disease compared with patients homozygous for the wild-type allele (p = 0.04; OR = 0.67; 95% CI = 0.37–0.95). No other significant associations were observed in Caucasian patients (Suppl Table 1). Thirty-five percent (13/37) of American Caucasian CMV disease patients carried LILRB1 I142T*(1) and IL10-1082*(1,2) compared with 19% (9/48) of controls, although this was not significant (p = 0.13; OR = 2.35; Fisher’s test).

### 3.3. Associations with nadir CD4 T cell counts

A preliminary analysis of the data indicated that African American cases had slightly lower nadir CD4 T cell counts than controls (median nadir CD4 T cell count [IQR] = 4 [10–11] vs 10 [3–30]; p = 0.06; Mann–Whitney). The same was observed in Caucasian patients (median nadir CD4 T cell count [IQR] = 10 [0–20] vs 15 [3–30]; p = 0.07; Mann–Whitney). However, this observation does not reveal whether the observed association with low nadir CD4 T cell count was a consequence of CMV disease. Hence, we examined genetic associations with nadir CD4+ T cell counts to shed light on the causes of CMV end-organ disease.

In African Americans, heterozygous and homozygous carriage of allele 2 at DARC T-46C conferred significantly higher nadir CD4 T cell counts compared with patients homozygous for allele 1 (p = 0.002; IRR = 4.1; 95% CI = 2.3–40; and p = 0.003; IRR = 8.5; 95% CI = 8.5; respectively; 95% CI = 0.1–0.81; Table 4a). However, patients who were homozygous for allele 2 of CD14 C(159)T had much lower nadir CD4 T cell counts than those homozygous for allele 1 (p = 0.01; IRR = 0.33; 95% CI = 0.14–0.81; Table 4a). Negative binomial regression modeling confirmed that homozygous carriage of allele 2 in DARCT-46C was the strongest determinant of low nadir CD4+ T cell count in African Americans (p = 0.009; data not shown). No other significant associations were observed in African Americans (Suppl Table 2).

Caucasians who were homozygous for allele 2 of TNFA-1031 had significantly lower nadir CD4 T cell counts compared with patients homozygous for allele 1 (p = 0.005; IRR = 0.06; 95% CI = 0.008–0.42; Table 4a). Carriage of allele 2 at IL2-330 was associated with higher nadir CD4 T cell counts compared with patients homozygous for allele 1 (p = 0.02; IRR = 2.4; 95% CI = 1.2–4.9; and p = 0.04; IRR = 4.1; 95% CI = 1.1–15; respectively; 95% CI = 0.14–0.96; Table 4a). Caucasians who were homozygous for allele 2 of CCL2-2518 had lower nadir CD4 T cell counts compared with patients homozygous for allele 1 (p = 0.02; IRR = 0.25; 95% CI = 0.08–0.77; Table 4a).

In Caucasians, heterozygosity in LILRB1 I142T was associated with higher nadir CD4 T cell counts compared with patients homozygous for allele 1 (p = 0.005; IRR = 0.06; 95% CI = 0.008–0.42; Table 4a). Bootstrap multivariable modeling confirmed TNFA-308, IL2-330, IL10-1082, LIL23’UTR, CCL2-2518, LILRB1 I142T affect nadir CD4 T cell counts in Caucasians (p = 0.006; p = 0.04; p = 0.03; p = 0.02; p = 0.04; respectively, where indicated in Table 4b). No other significant associations were found with the remaining polymorphisms in Caucasians (Suppl Table 2).

### 3.4. Associations with CMV disease and nadir CD4 T cell counts

We then performed logistic regression for each SNP adjusting for nadir CD4 T cell count. However, nadir CD4 T cell count had no impact on the relationship between CMV disease and the SNP for...
Table 4a
Inflammation, immune-related single nucleotide polymorphisms (SNPs), and polymorphisms associated with natural killer cell activity associated with low nadir CD4⁺ T cell counts

<table>
<thead>
<tr>
<th>Gene alias/SNP</th>
<th>Genotype</th>
<th>African Americans (n = 155)</th>
<th>Caucasians (n = 85)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Median nadir count</td>
<td>IQR</td>
</tr>
<tr>
<td>TNFA-1031</td>
<td>CC</td>
<td>66 9 1–30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>30 10 2–27</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>4 9 0–32</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>CT/TT</td>
<td>34 10 1–27</td>
<td>0.87</td>
</tr>
<tr>
<td>IL2-330</td>
<td>TT</td>
<td>86 10 0.5–32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>14 1 1.5–49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG/GG</td>
<td>14 7 1.5–49</td>
<td>1.54</td>
</tr>
<tr>
<td>CCL2-2518</td>
<td>AA</td>
<td>63 10 2.2–29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>34 10 0.6–26</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>3 9 0.5–22</td>
<td>0.33</td>
</tr>
<tr>
<td>DARC T-46C</td>
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<td>5 1 0–10</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>31 10 1–26</td>
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</tr>
<tr>
<td></td>
<td>GG</td>
<td>64 10 0.75–30</td>
<td>8.5</td>
</tr>
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<td>CD14 (−159)T</td>
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</tr>
<tr>
<td></td>
<td>CT</td>
<td>40 10 1–26</td>
<td>0.86</td>
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<td></td>
<td>TT</td>
<td>13 4 0–20</td>
<td>0.33</td>
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<td>LILRB1 I142T</td>
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<td>0.73</td>
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<td>TC</td>
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<tr>
<td></td>
<td>TC/CC</td>
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<td>1.11</td>
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<td></td>
<td>TC/CC</td>
<td>31 6 1–24</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>CC/CC</td>
<td>79 10 2–19</td>
<td>0.99</td>
</tr>
</tbody>
</table>

95% CI, 95% confidence interval; IRR, incident rate ratio.
Significant p values < 0.05 indicated in bold.
*Percentage frequency of carriage.
Interquartile range (25–75%).
*p value calculated by negative binomial regression.

African Americans (p = 0.13–0.33) or Caucasians (p = 0.07–0.60; data not shown).

4. Discussion

Our earlier study of Caucasian Australian HIV patients indicated that 92% of HIV patients with herpesvirus IRD were homozygous for carriage of the wild-type allele in IL12B3 UTR [5]. The same allele was associated with lower nadir CD4⁺ T cell counts, suggesting that susceptibility to CMV disease may be the consequence of a genetic determinant for low CD4⁺ T cell counts. In the current study African American patients homozygous for the wild-type allele in IL12B3 UTR had marginally lower nadir CD4⁺ T cell counts (p = 0.07, OR = 0.53; Suppl Table 2) and a greater frequency of CMV disease compared with patients with allele 2. However, this finding was not evident in Caucasians—perhaps Caucasian Australians are predominantly Northern European, whereas the population in Cleveland may be more diverse. The IL12B gene encodes IL-12p40, which is a component of both IL-12 and IL-23. The IL12B3 UTR SNP has been associated with increased IL-12 production [21], so the converse is true of homozygous carriage of the wild-type allele. Low IL-12 production by homozygous carriers of the wild-type allele will affect enhancement of cytotoxic activity of NK cells and CD8⁺ T cells. It may also affect differentiation of naive T cells into Th0 cells and the growth and function of T cells. This finding offers mechanisms by which the SNP may affect CD4⁺ T cell counts. The effect of the allele on levels of IL-23 and p40 homodimer (which has a suppressive role) is unclear.

In the study noted above, TNFA-308*2 was carried by 52% of HIV patients who experienced IRD associated with a herpesvirus infection, because several patients with exacerbations of CMV retinitis carried this allele as part of the 44.1 ancestral haplotype ([HLA-A2, B44, TNFA-308*2, BAT1(intron10)*1, DR3] [5]). In the current study only 32% of Caucasian patients with a history of CMV disease carried the variant allele compared with 19% in the CMV-seropositive control cohort, but this finding was not significant (p = 0.17; Suppl Table 1). However, when the Caucasian patients were pooled to examine associations with low nadir CD4⁺ T cell counts, TNFA-308*2 was significant in the overall multivariate model (p = 0.006; Table 4b). SNPs in genes nearby or in linkage disequilibrium with TNFA-308*2 may also influence these phenotypes. For example, TNFA-308*2 affects neuropathy in Malays and Chinese patients [22]. TNFA-308*2 and TNFA-1031*2 lie in distinct “TNF block haplotypes” within the central MHC [23]. MHC haplotypes are defined by HLA and TNF alleles and are associated with susceptibility to numerous inflammatory diseases, such as rheumatoid arthritis, type 1 diabetes, and sepsis [24–26].

IL10-1082*2(G) was associated with CMV disease in Caucasian HIV patients. This association has been reported after allogeneic stem cell [27] and renal [28] transplantation in Caucasian cohorts. Homozygous carriage of allele 2 was associated with high expres-
sion of IL-10 transcript in Caucasian patients [29]. In Italian centenarians, the IL10-1082(2,2) genotype was associated with high IL-10 production [30]. Similarly, allele 2 was associated with elevated IL-10 production in peripheral blood leukocytes [31]. CMV encodes a homologue of human IL-10, which may contribute to evading immune surveillance [32,33]. This supports the model where a polymorphism in IL10 is favorable for the propagation of CMV through higher expression of IL-10. An increase in IL-10 may favor anti-inflammatory pathways, which may have adverse consequences [34].

In Australian Caucasian HIV patients, carriage of more activating KIR was associated with CMV disease [11]. However, this finding is not evident in the subset of African Americans tested. Carriage of the wild-type allele tested in LIRBL1142T affected CMV disease and nadir CD4+ T cell counts separately in Caucasians, supporting a role for the encoded protein (LIR-1) in CMV and HIV disease. The SLC11A1 gene encodes a divergent transition metal (Fe2+, Zn2+, and Mn2+) transporter membrane protein and affects immune reactions to intracellular pathogens [35]. In African Americans, carriage of allele 2 at SLC11A1 D543N was associated with CMV disease. This is consistent with associations between SLC11A1 D543N and susceptibility to inflammatory diseases, such as Crohn’s disease in Greek patients [36] and Buruli ulcers in Ghanaian patients [37].

Duffy antigen receptor chemokine (DARC) is expressed on red blood cells and can affect plasma levels of proinflammatory chemokines such as CCL5/RANTES, which can inhibit HIV replication [14]. Hence, DARC could affect immune activation and HIV pathogenesis. Allele 2 at DARC T-46C confers a DARC-negative phenotype. This has been associated with protection among individuals of African descent against Plasmodium vivax malaria infection [38,39]. Recently, this phenotype was also associated with susceptibility to HIV infection and rate of progression to AIDS [40]. HIV-1 attaches to red blood cells via DARC, mediating trans-infection of PBMC or NP2 cells that express CD4 and either CXCR4 or CCR5. Following HIV infection, the DARC-negative red blood cell phenotype was associated with slower disease progression [40]. Similarly, in the current study, homozygous carriage of allele 1 in DARC T-46C was associated with low nadir CD4+ T cell counts, consistent with a role in accelerated HIV disease progression. Other studies have generated conflicting results, however, [41–44], so the issue awaits multivariable analyses that factor in comorbidities and standardize the assessment of disease progression.

Overall in African Americans, DARC T-46C was the clearest determinant of low nadir CD4+ T cell counts, with progressively smaller effects of CD14C 159T and IL12B 3’UTR. Of these, only IL12B 3’UTR was associated with CMV disease. In Caucasians, alleles of several immune-related genes [TNFA-308, IL2-330, IL10-1082, IL12B 3’UTR, CCL2-2518, and LIRBL1142T] were associated with low nadir CD4+ T cell counts, with LIRBL1142T and IL10-1082 also affecting CMV disease. The effects of IL12B 3’UTR, LIRBL1142T, and IL10-1082 on levels of IFN-γ, IL-1, and IL-10, as well as on NK cell function, warrant investigation in this context.

Acknowledgments
The authors would like to acknowledge the support of the clinical core of the Case Western Reserve University (CRWU) Center for AIDS Research AI-36219.

Appendix. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.humimm.2011.11.005.

References


**Supplementary Table 1: Polymorphisms not associated with CMV disease in African American and Caucasian cohorts.**

<p>| Gene Alias/SNP | Genotype | African Americans | | | | Caucasians | | | |
|---|---|---|---|---|---|---|---|---|
| | | Controls (n=114) | Cases (n=41) | OR | 95% CI | P | Controls (n=48) | Cases (n=37) | OR | 95% CI | P |
| <strong>Inflammation and immune-related SNPs</strong> | | | | | | | | | | | |
| TNFA-308 (G/A) | GG | 78 | 78 | 1.1 | 0.4-2.8 | 1 | 81 | 68 | 1.7 | 0.7-5.5 | 0.17 |
| | GA | 20 | 22 | 1.2 | 0.15-13 | 1 | 17 | 30 | 2 | |
| | AA | 2 | 0 | 1 | 0-15 | 1 | 2 | 2 | 1 | |
| | GA/AA | 22 | 22 | 1 | 0.4-2.5 | 1 | 19 | 32 | 2.0 | 0.7-5.5 | 0.17 |
| TNFA-1031 (C/T) | CC | 66 | 68 | 0.8 | 0.3-1.8 | 0.51 | 64 | 51 | 1.7 | 0.7-4.2 | 0.23 |
| | CT | 32 | 24 | 3 | 0.6-16 | 0.19 | 5 | 46 | 1.7 | 0.7-4.2 | 0.23 |
| | TT | 2 | 8 | 0.9 | 0.4-2.0 | 0.84 | 31 | 3 | 1.7 | 0.7-4.2 | 0.23 |
| | CT/TT | 34 | 32 | 0.9 | 0.4-2.0 | 0.84 | 36 | 49 | 1.7 | 0.7-4.2 | 0.23 |
| BAT1 (intron 10) | -- | 84 | 83 | 1.2 | 0.4-3.3 | 0.93 | 85 | 84 | 1.7 | 0.7-4.2 | 0.23 |
| | -C | 15 | 17 | 2.8 | 0.1-111 | 1 | 15 | 13 | 1.7 | 0.7-4.2 | 0.23 |
| | CC | 1 | 0 | 1.1 | 0.4-2.9 | 0.85 | 0 | 3 | 1.7 | 0.7-4.2 | 0.23 |
| | -C/CC | 16 | 17 | 1.1 | 0.4-2.9 | 0.85 | 15 | 16 | 1.1 | 0.3-3.7 | 0.84 |
| IL1A+4845 (C/A) | CC | 71 | 66 | 1.2 | 0.6-2.6 | 0.62 | 50 | 49 | 1.7 | 0.7-4.2 | 0.23 |
| | CA | 28 | 32 | 3 | 0.2-49 | 0.44 | 44 | 46 | 1.7 | 0.7-4.2 | 0.23 |
| | AA | 1 | 2 | 1.3 | 0.6-2.7 | 0.53 | 6 | 5 | 1.7 | 0.7-4.2 | 0.23 |
| | CA/AA | 29 | 34 | 1.3 | 0.6-2.7 | 0.53 | 50 | 51 | 1.7 | 0.7-4.2 | 0.23 |
| IL2-330 (T/G) | TT | 88 | 80 | 1.9 | 0.7-4.9 | 0.20 | 49 | 58 | 1.7 | 0.7-4.2 | 0.23 |
| | TG | 12 | 20 | 1.9 | 0.7-4.9 | 0.20 | 38 | 39 | 1.7 | 0.7-4.2 | 0.23 |
| | GG | 13 | 3 | 0.8 | 0.3-2.1 | 0.73 | 13 | 3 | 1.7 | 0.7-4.2 | 0.23 |
| | TG/GG | 51 | 42 | 0.7 | 0.3-1.6 | 0.40 | 51 | 42 | 1.7 | 0.7-4.2 | 0.23 |
| IL4-589 (T/C) | CC | 12 | 10 | 1.5 | 0.4-5.2 | 0.52 | 67 | 75 | 1.7 | 0.7-4.2 | 0.23 |
| | CT | 38 | 46 | 1.1 | 0.3-3.7 | 0.92 | 33 | 25 | 1.7 | 0.7-4.2 | 0.23 |
| | TT | 50 | 44 | 1.1 | 0.3-3.7 | 0.92 | 51 | 42 | 1.7 | 0.7-4.2 | 0.23 |
| IL18-137 (G/C) | GG | 65 | 68 | 0.7 | 0.3-1.6 | 0.36 | 56 | 54 | 1.7 | 0.7-4.2 | 0.23 |
| | GC | 31 | 22 | 2.6 | 0.6-11 | 0.20 | 42 | 41 | 1.7 | 0.7-4.2 | 0.23 |
| | CC | 4 | 10 | 2.6 | 0.6-11 | 0.20 | 2 | 5 | 2.7 | 0.2-3.2 | 0.43 |
| | GC/CC | 35 | 32 | 0.9 | 0.4-1.9 | 0.72 | 44 | 46 | 1.7 | 0.7-4.2 | 0.23 |</p>
<table>
<thead>
<tr>
<th>Polymorphism associated with Natural Killer cell activity</th>
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<tr>
<td><strong>IL18-607</strong></td>
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<tr>
<td>TT</td>
</tr>
<tr>
<td>TG</td>
</tr>
<tr>
<td>GG</td>
</tr>
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<td>TG/GG</td>
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<tr>
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<td><strong>HLA-G -/+14 indel</strong></td>
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</table>

| a | % Frequency of carriage |
| b | Odds ratio |
| c | 95% confidence interval |
| d | P value calculated by logistic regression |
| e | P value calculated by exact logistic regression (where n is small in respective category) |
| f | median unbiased estimates |
**Supplementary Table 2:** Polymorphisms *not* associated with low nadir CD4⁺ T cell counts in African American and Caucasian donors.

<table>
<thead>
<tr>
<th>Gene Alias/SNP</th>
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<th>Caucasians (n=85)</th>
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<td>median nadir count</td>
<td>intq range</td>
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<td>Gene</td>
<td>Frequency Carriage</td>
<td>Interquartile Range</td>
<td>Incident Rate Ratio (IRR)</td>
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<td>--------------------</td>
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<td>VDR FokI (F/f)</td>
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<tr>
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<td>57</td>
<td>0.5 - 29</td>
<td>41</td>
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<tr>
<td>CT</td>
<td>38</td>
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<td>TT</td>
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<td>8</td>
<td>14</td>
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<tr>
<td>CT/TT</td>
<td>43</td>
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<tr>
<td>SLC11A1 D543N</td>
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<tr>
<td>AA</td>
<td>87</td>
<td>1 - 28</td>
<td>95</td>
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<td>AG</td>
<td>13</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>AG/GG</td>
<td>13</td>
<td>0 - 20</td>
<td>5</td>
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**Polymorphism associated with Natural Killer cell activity**

<table>
<thead>
<tr>
<th>HLA-G -/+14 indel</th>
<th>Frequency Carriage</th>
<th>Interquartile Range</th>
<th>Incident Rate Ratio (IRR)</th>
<th>95% Confidence Interval</th>
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<td>--</td>
<td>38</td>
<td>3-22.5</td>
<td>38</td>
<td>12</td>
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<tr>
<td>-/+</td>
<td>47</td>
<td>0-28</td>
<td>1.2</td>
<td>0.65 – 2.3</td>
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<tr>
<td>++</td>
<td>15</td>
<td>0-24</td>
<td>1.0</td>
<td>0.41 – 2.4</td>
</tr>
<tr>
<td>-/+++</td>
<td>62</td>
<td>0-28</td>
<td>1.2</td>
<td>0.64 – 2.1</td>
</tr>
</tbody>
</table>

a % Frequency of carriage
b Interquartile range (25%-75%)
c Incident rate ratio (IRR)
d 95% confidence interval
e 95% confidence interval
f P value calculated by negative binomial regression
Chapter 6

The immunological footprint of CMV in HIV-1 patients stable on long-term ART


This chapter is presented in the form as published online and constitutes pages 58-65 in this thesis.

Chapter outline:

This chapter focuses on investigating the immunological “footprint” of CMV on the immune system of CMV seropositive HIV patients who have been on ART for more than 12 years, via its effects on T cell senescence.
The immunological footprint of CMV in HIV-1 patients stable on long-term ART

Jacquita S. Affandi¹,², Jacinta Montgomery¹, Samantha J. Brunt¹, David Nolan³ and Patricia Price²*

Abstract

Background: Most HIV-infected persons are cytomegalovirus (CMV) seropositive and retain latent virus that can be reactivated by immune activation. Their T cell populations express markers reflecting a late stage of differentiation, but the contributions of HIV and CMV to this profile are unclear. We investigated the immunological "footprint" of CMV in HIV patients who had a history of extreme immunodeficiency but were now stable on antiretroviral therapy (ART).

Results: Twenty CMV seropositive HIV patients >50 years old with nadir CD4 T-cell counts <200 cells/μl were studied after >12 years on ART. 16 CMV seropositive and 9 CMV seronegative healthy controls were included. CMV antibody titres were higher in HIV patients than controls (P < 0.001-0.003). Levels of soluble B-cell activating factor (sBAFF) were elevated in patients (P = 0.002) and correlated with levels of CMV antibodies (P = 0.03-0.002), with no clear relationship in controls. CD8 T-cell IFNγ responses to the IE1 peptide (VLE) remained elevated in HIV patients (P = 0.005). The CD57⁺CD45RA⁺CD27⁻ phenotype of CD8 T-cells correlated with age (r = 0.60, P = 0.006), antibodies against CMV IE1 protein (r = 0.44, P = 0.06) and CD4 T-cell IFNγ response to CMV lysate (r = 0.45, P = 0.05).

Conclusions: Humoral and T-cell responses to CMV remained elevated in HIV patients after >12 years on ART. Age and presence of CMV disease influenced CD8 T-cell phenotypes. Elevated levels of sBAFF may be a consequence of HIV disease and contribute to high titres of CMV antibody.

Keywords: HIV, CMV, ART, Immunosenescence, Age

Background

Cytomegalovirus (CMV) infections may be asymptomatic or cause mild symptoms in immunocompetent hosts, but can cause morbidity and mortality in human immunodeficiency virus -1 (HIV) patients. CMV end-organ disease is common in patients with low CD4-T-cell counts, but long term consequences are less clear. At any time, immune activation may promote the reactivation of CMV leading to the re-stimulation of CMV-specific T-cells [1]. This creates T-cell populations enriched with differentiated, apoptosis-resistant memory T cells with limited proliferative capabilities, and leaves an immune system with limited capacity to recognize novel antigens [2]. In the elderly people not infected with HIV, CMV infection has been linked with accelerated immune ageing and/or immunosenescence [3–5], with increased risk for mortality and age-related morbidities [2].

It is reasonable to hypothesize that CMV and other coinfections may contribute to the “accelerated ageing” syndrome observed in HIV-infected individuals [1, 6]. CMV coinfection has been associated with an increased risk of severe non-acquired immune deficiency syndrome (AIDS)-defining events in HIV-infected patients [7]. Untreated HIV infection and chronological ageing are similarly associated with many T cell abnormalities [8, 9]. This includes low CD4/CD8 ratios, low naïve/memory T cell ratios, reduced T cell repertoire, and an expansion of CD57⁺ T cells. CD57 expression can be used to monitor proliferative history, poor proliferative capacity [9], replicative senescence and antigen-induced apoptotic death [10]. Memory T-cells that have undergone multiple rounds of restimulation can also be characterized phenotypically by re-expression of CD45RA [11] and the absence of CD27 [12]. Persistent viral infections, inflammatory syndromes and ageing induce the accumulation of...
highly differentiated memory T cells re-expressing CD45RA [13]. In HIV infected children and CMV-seropositive healthy children [14], the frequency of CD45RA⁺CD27⁻ phenotype on CD8 T cells correlated with previous CMV infection as measured by serum immunoglobulin G (IgG) levels against CMV. Here we address which CD4 and CD8 T cell markers best define the T-cell phenotype associated with a high burden of CMV in older HIV patients stable on combination antiretroviral therapy (ART).

HIV-seronegative, CMV-seropositive individuals who control CMV replication have very high frequencies of CMV-specific CD8 T-cells able to respond to multiple CMV proteins [15]. Proportions of CMV reactive CD8 T-cells rise rapidly with age in HIV infected patients [16]. The CMV proteins pp65, glycoprotein B (gB) and Immediate Early-1 (IE1) [17] are targets of the CD8 T-cell response against CMV. The peptides NLVPMVATV ["NLV" from CMV pp65] and VLETSVML ["VLE" from CMV IE1] evoke easily measurable CD8-T-cell responses in healthy CMV-seropositive individuals carrying human leucocyte antigen (HLA)-A*02 [18]. Stone et al. showed that responses to VLE were elevated in previously immunodeficient HIV patients stable on ART when compared to controls [19]. IE1 is expressed early during CMV replication so these cells may be important for protection against CMV reactivation from latency.

Levels of monocyte and B-cell activation are elevated in untreated HIV patients and may remain high on ART [20]. Markers of monocyte activation and tumour necrosis factor (TNF) activity include soluble TNF receptor 1 (sTNFR1) [21] and soluble CD14 (sCD14) [22]. B cell activation can be gauged through levels of total IgG and soluble B-cell activating factor (sBAFF) [23]. BAFF is a novel member of the TNF ligand family and plays an important role in B lymphocyte maturation and survival. BAFF is involved in the pathogenesis of several autoimmune disorders [24] and with risk of long-term kidney graft dysfunction [25].

With the increased availability of ART worldwide, large numbers of patients begin ART with advanced HIV disease and live for many years. This present study investigates T-cell changes as a "footprint" of CMV in a unique cohort of older HIV patients who began ART with advanced immunodeficiency more than twelve years previously and have maintained viral suppression for more than a year. We measured levels of antibodies to CMV lysate, CMV IE1 and CMV gB, using extensive titrations to ensure quantitation in the high range. As titres of all three antibodies were elevated in the HIV patients, we sought explanations for the increase. This included the assessment of sTNFR1, sCD14, total IgG and sBAFF, as well as host interferon gamma (IFNγ) responses of CD4 and CD8 T-cells to CMV proteins.

Results

CMV lysate, CMV gB and CMV IE1 antibody titres are higher in HIV patients than healthy controls and more tightly correlated

The study focuses on the long term outcome for CMV seropositive HIV patients stable on ART. HIV patients presented with a median nadir CD4 T-cell count of 78 cells/μL (range: 0–195). Eleven of the 20 HIV patients and 9 out of 16 CMV seropositive (CMV⁺) controls carried the HLA-A*02 allele, but no CMV seronegative (CMV⁻) controls were carriers. HIV patients had been on ART for more than 12 years [174 (159–189) months] with a median CD4 T-cell count of 691 cells/μL (range: 372–1848) at time of testing.

HIV patients and CMV⁺ controls were similar in age, but CMV⁻ controls were slightly younger (Table 1). Levels of antibodies reactive with CMV lysate, CMV gB and CMV IE1 were higher in HIV patients than CMV⁺ controls (Table 1). In HIV patients, levels of antibodies reactive with different CMV antigens were tightly correlated (lysate vs gB, r = 0.81, P < 0.0001; lysate vs IE1, r = 0.70, P = 0.0005; gB vs IE1, r = 0.57, P = 0.009). In CMV⁺ controls, similar trends were evident (lysate vs gB, r = 0.57, P = 0.02; lysate vs IE1, r = 0.52, P = 0.04) although CMV gB and CMV IE1 did not differ (r = 0.17, P = 0.53).

High CMV antibody levels in HIV patients may reflect increased exposure to CMV antigens before they began ART, but could also indicate persistent B-cell activation. Hence, we assessed levels of sBAFF and total IgG to examine whether antibody levels reactive with CMV reflect polyclonal B-cell activation.

B-cell activation (sBAFF and IgG), but not monocyte activation (sCD14 and sTNFR1), may contribute to high CMV antibody titres in HIV patients

HIV patients had higher levels of sBAFF than CMV⁺ controls (Table 1). There was a direct relationship between CMV antibodies and sBAFF in patients [CMV lysate (r = 0.72, P = 0.002), CMV gB (r = 0.70, P = 0.003), CMV IE1 (r = 0.54, P = 0.03)]. However in CMV⁺ controls, a weak inverse relationship was observed between levels of sBAFF and CMV lysate (r = −0.47, P = 0.08) and CMV IE1 (r = −0.51, P = 0.06). In HIV patients, sBAFF levels correlated with levels of total IgG (r = 0.70, P = 0.003), but this was not seen in CMV⁺ controls (r = −0.53, P = 0.04).

Levels of total IgG in HIV patients correlated with antibodies to CMV gB (r = 0.65, P = 0.002) and CMV lysate (r = 0.40, P = 0.08) however these observations were
Table 1 HIV patients stable on ART retain elevated humoral and cellular responses to CMV and advanced T-cell senescence

<table>
<thead>
<tr>
<th></th>
<th>HIV patients</th>
<th>CMV + Controls</th>
<th>CMV- Controls</th>
<th>A vs B</th>
<th>A vs C</th>
<th>B vs C</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>16</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male:Female</td>
<td>19:1</td>
<td>14:2</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>62.5 (50–73)</td>
<td>60 (50–74)</td>
<td>55 (52–69)</td>
<td>0.19</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>Levels in Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV lysate antibody AU/L</td>
<td>94 (23–995)</td>
<td>20 (6–83)</td>
<td>0.8 (0.5–1.1)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CMV gB antibody AU/L</td>
<td>127 (27–400)</td>
<td>45 (2–88)</td>
<td>2 (0.6-3.3)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CMV IE-1 antibody AU/L</td>
<td>49 (8–1098)</td>
<td>9 (2–180)</td>
<td>2.6 (1.9–10)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.003</td>
</tr>
<tr>
<td>sCD14 ng/mL</td>
<td>22 (7.7–48)</td>
<td>18 (8.3–39)</td>
<td>22 (11–31)</td>
<td>0.09</td>
<td>0.23</td>
<td>0.68</td>
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<tr>
<td>sTNFR1 pg/mL</td>
<td>15 (9.1–25)</td>
<td>15 (12–27)</td>
<td>17 (10–21)</td>
<td>0.68</td>
<td>0.83</td>
<td>0.72</td>
</tr>
<tr>
<td>Total IgG mg/mL</td>
<td>12 (3–22)</td>
<td>12 (5–20)</td>
<td>94 (6.5–15)</td>
<td>0.46</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>sBAFF ng/mL</td>
<td>740 (376–1401)</td>
<td>519 (274–786)</td>
<td>319 (318–471)</td>
<td>0.002</td>
<td>0.0003</td>
<td>0.06</td>
</tr>
<tr>
<td>IFNγ spots per 2 × 10⁶ cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV lysate</td>
<td>227 (16–700)</td>
<td>157 (13–617)</td>
<td>0 (0–0.5)</td>
<td>0.16</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CMV pp65</td>
<td>445 (14–1591)</td>
<td>138 (18–645)</td>
<td>0 (0–2)</td>
<td>0.005</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CEF control peptide pool</td>
<td>516 (2–1500)</td>
<td>337 (20–584)</td>
<td>4 (0–404)</td>
<td>0.07</td>
<td>0.001</td>
<td>0.0015</td>
</tr>
<tr>
<td>NLV peptide</td>
<td>498 (56–1363)</td>
<td>214 (13–651)</td>
<td>na</td>
<td>0.06</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>VLE peptide</td>
<td>420 (14–2000)</td>
<td>25 (7–561)</td>
<td>na</td>
<td>0.005</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

| T cell subset [as % of] |          |                |               |        |        |        |
| CD4⁺ T cells [lymphocytes] | 43 (24–77) | 69 (52–84)     | 69 (52–80)    | <0.0001 | 0.0009 | 0.93   |
| CD57⁺ [CD4]          | 11 (2–75)  | 8 (2–26)       | 4.5 (1–7.4)   | 0.11   | 0.001  | 0.03   |
| CD57⁺CD45RA⁺CD27⁻ [CD4] | 1.9 (0–57) | 0.44 (0.06–14)| 0.02 (0.005–0.16) | 0.06   | 0.0002 | 0.0001 |
| CD8⁺ T cells [lymphocytes] | 48 (16–71) | 22 (7–43)     | 21 (15–44)    | 0.0001  | 0.002  | 0.07   |
| CD57⁺ [CD8]          | 47 (17–67) | 40 (6.4–69)   | 28 (10–68)    | 0.08   | 0.04   | 0.51   |
| CD57⁺CD45RA⁺CD27⁻ [CD8] | 19 (4.2–53) | 26 (4–49) | 8 (2–19) | 0.61   | 0.03   | 0.0001 |

na = Not applicable as none of the CMV-seronegative healthy controls carried the HLA-A*02 allele

*Median (range)

Mann–Whitney, P ≤ 0.05 (bold), P > 0.05-0.1 (italics)

HLA-A*02 allele restricted thus HIV+ patients n = 11

CMV+ controls n = 9

not evident in CMV+ controls (r = 0.09 to 0.30, P = 0.26 to 0.74).

Levels of sCD14 and sTNFR1 were similar in all groups (Table 1). In CMV+ controls, sCD14 levels correlated inversely with CMV antibodies (CMV lysate, r = −0.50, P = 0.05; CMV gB, r = −0.51, P = 0.05; CMV IE1, r = −0.49, P = 0.06), whilst these parameters were unrelated in patients (r = 0.04 to 0.35, P = 0.13 to 0.86). sTNFR1 levels did not correlate with antibodies to CMV antigens in any group.

IFNγ responses to the CMV IE1 peptide (VLE) remain elevated in HIV patients

IFNγ responses were assessed by enzyme linked immunosorbent spot assay (ELISpot) in peripheral blood mononuclear cells (PBMC) stimulated with whole CMV lysate (mediated by CD4 T-cells), CMV pp65 peptide pool (mediated by CD4 and CD8 T-cells), CMV, EBV and influenza (CEF) control peptide pool (mediated by CD8 T-cells), and HIV-A*02 restricted CMV peptides (VLE and NLV; mediated by CD8 T-cells) [26].

HIV patients had more CD4 and CD8 T-cells producing IFNγ in response to CMV pp65 peptide pool (P = 0.005, Table 1) and more CD8 T-cells producing IFNγ in response to VLE peptide (P = 0.005) than CMV+ controls. However, numbers of CD4 T-cells responding to CMV lysate were similar in patients and CMV+ controls (Table 1) and responses to the CEF peptide pool were only marginally lower in CMV+ controls (Table 1). This had been described in patients from our clinic tested after 4 years on ART and 6 months of complete viral suppression [19]. As expected, CMV- controls had low/undetectable IFNγ responses to CMV lysate (Table 1), CMV pp65 peptide pool (Table 1) or even CEF control peptide pool (P = 0.001) (Table 1).
In HIV patients, expression of CD57+CD45RA−CD27+ on CD8 T-cells correlated with age, CMV IE1 and CD4 T-cell IFNγ responses to CMV lysate

Proportions of CD8 T-cells in HIV patients were higher than in controls (Table 1), but the CD57+ or CD57+CD45RA−CD27+ phenotypes of CD8 T-cells were equally common in HIV patients and CMV+ controls (Table 1). However CMV- controls had few CD8 T-cells expressing CD57+CD45RA−CD27+, so the accumulation of these cells is driven by CMV.

In HIV patients, proportions of CD8 T-cells with highly differentiated phenotypes (CD57+ or CD57+CD45RA−CD27+) correlated with age (r = 0.67, P = 0.001; r = 0.60, P = 0.006, resp), antibodies reactive with CMV IE1 (r = 0.44, P = 0.06; r = 0.41, P = 0.07) and IFNγ responses to CMV lysate (r = 0.45, P = 0.05; r = 0.42, P = 0.07). Expression of CD57+ on CD8 T-cells was also correlated with IFNγ responses to the CMV IE1 VLE peptide (n = 9; r = 0.60, P = 0.05) and age (r = 0.67, P = 0.001). CMV+ controls did not show equivalent correlations.

HIV patients retained lower proportions of CD4 T-cells than CMV+ or CMV- controls (Table 1). Proportions of CD4 T-cells with the phenotype CD57+CD45RA−CD27+ were marginally higher in HIV patients than in CMV+ controls (Table 1). CMV+ controls had more CD57+ or CD57+CD45RA−CD27+ CD4 T-cells than CMV- controls so again CMV is the driving force for CD4 T-cell differentiation (Table 1). Accordingly in patients, CD57 expression on CD4 T-cells correlated weakly with antibody and IFNγ responses to CMV lysate (r = 0.41, P = 0.07; r = 0.39, P = 0.09).

**Discussion**

CMV seropositivity has been placed in an “immune risk profile” associated with mortality in longitudinal studies of people over 85 years old [3, 5]. Here, we describe investigations of a unique cohort of CMV seropositive HIV patients over 50 years of age who began ART with advanced immune deficiency over 12 years earlier and maintained undetectable plasma HIV RNA for more than a year. Their levels of antibodies reactive with CMV lysate, gB and IE1 remained elevated despite the long period on effective ART.

Elevated sBAFF levels have been associated with autoimmune diseases [27], graft versus host disease [28], and complications of kidney transplantation [29]. In CMV-deoxyribonucleic acid (DNA) positive renal transplant patients, sBAFF levels were higher than in CMV-DNA negative recipients, with positive correlations between CMV-DNA levels, total IgG and sBAFF [30]. Here, levels of sBAFF and total IgG correlated with antibodies reactive with CMV lysate, CMV gB and CMV IE1 in HIV patients. This suggests that B-cell activation is a feature of HIV disease and contributes to elevated titres of CMV antibodies. Although we found no associations between CMV antibodies and levels of sTNFR1 or sCD14, this may be apparent in extended studies that include HIV+CMV- patients and assess CMV-DNA. HIV+CMV- patients are rare so collaborative studies will be needed.

HIV+CMV+ patients had higher frequencies of CD8 T-cells specific for the CMV IE1 VLE peptide than CMV+ healthy controls, whilst frequencies of CD8 T-cells specific for the CMV pp65 NLY peptide were less clearly elevated, as expected [19]. IE1 proteins are expressed before pp65 during CMV reactivation, thus HIV patients on ART may experience CMV reactivation more frequently than controls. IFNγ responses to CMV pp65 peptide pool and CMV IE1 protein were also elevated in subjects >85 years old not infected with HIV [31]. No HIV patients displayed symptoms of CMV disease at the time PBMC were collected, so VLE-specific CD8 T-cells may play a role in averting CMV replication.

Elevated CD8 T-cell IFNγ responses to VLE may reflect oligoclonal expansion of CMV-specific CD8 T-cells before ART, as many patients in this study had a history of CMV end-organ disease. Long-term ART does not increase the diversity of T-cell repertoires in HIV patients despite sustained suppression of viral replication [32].

As CMV seropositivity has been linked to “accelerated ageing” [1], highly differentiated effector memory CD4 and CD8 T-cells were assessed by expression of CD57 and CD45RA without CD27 [12]. Proportions of CD8 T-cells with the phenotype CD57+CD45RA−CD27+ correlated with age and with antibodies against the CMV IE1 protein and CD4 T cell IFNγ response to CMV lysate. Thus demonstrates that CMV seropositivity may accelerate CD8 T-cell differentiation and further worsen the impairment caused by HIV.

HIV patients had marginally more highly differentiated CD4 T-cells than CMV+ controls. Proportions of CD4 and CD8 T-cells from HIV patients expressing CD57 correlated with IFNγ responses to CMV antigen and peptide (respectively), consistent with CD57+ T-cells selectively retaining the capacity to produce IFNγ [26].

**Conclusions**

Overall these results establish that the immunological “footprint” of CMV remains elevated in HIV patients after more than 12 years on ART and correlates with CD8 T-cell phenotypes. Elevated levels of sBAFF may be an effect of HIV and contribute to high titres of CMV antibody. Extension of our study in larger cohorts should include CMV-seronegative HIV patients and CMV-DNA assessments to further elucidate the mechanisms by which CMV reactivation accelerates immune ageing in HIV patients.
Methods

Patient and control cohorts
Twenty CMV-seropositive HIV patients were selected from the HIV database of the Department of Clinical Immunology and Immunogenetics, Royal Perth Hospital, Western Australia. Participants were all >50 years old with nadir CD4 T-cell counts < 200 cells/ul, studied after >12 years on ART and >1 year of complete viral suppression (<50 HIV RNA copies/ml). All participants were healthy at the time of sample donation. ART comprised at least three antiretroviral drugs including a non-nucleoside reverse transcriptase inhibitor or protease inhibitor. Sixteen CMV-seropositive healthy controls (designated CMV+ controls) and nine CMV-seronegative healthy controls (designated CMV- controls) were included. Seropositivity was defined as >1100 AU/mL of CMV lysate antibody, where the cut-off was based on eleven samples from persons confirmed to be CMV seronegative by routine serology (Abbott Diagnostic Systems, Lake Forrest, IL, USA). Informed consent was obtained from all participants, and the human experimentation guidelines of Royal Perth Hospital and University of Western Australia were followed. Samples were screened for carriage of HLA-A*02 by flow cytometry [19]. Eleven of 20 (55 %) patients, nine of 16 (56 %) CMV+ controls and no CMV- controls, carried HLA-A*02.

T-cell subset phenotyping
Freshly collected PBMC [isolated by ficoll density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare Biosciences, Sweden)] were stained for surface markers (15 min, room temperature) using conjugated monoclonal antibodies as follows: CD8-FITC (clone SK1), HLA-DR-PE (clone TU36), CD57-APC (clone NK-1), CD45RA-APC-H7 (clone HI100), CD27-PerCPCy5.5 (clone M-T271), CD3-V450 (clone UCHT1) and CD4-V500 (clone RPA-TA) from BD Biosciences (San Jose, CA, USA). Stained cells were washed twice and analysed on a FACSCantoTM (BD Biosciences). At least 200 000 events were acquired and analysed using FlowJo (Treestar, San Carlos, CA, USA). Expression of CD57, CD27 and CD45RA was used to define terminally differentiated effector memory (TEMRA) (CD57+CD45RA+CD27+) T-cells. For comparisons with other studies, we also identified senescent T-cells solely by expression of CD57. Gating strategies can be found in Additional file 1: Figure S1.

Immune activation and total IgG ELISA
Levels of sTNFR1, sCD14 (RnD Systems; Minneapolis, MI, USA) and sBAFF (Abcam; Cambridge, UK) in plasma were quantified using commercial reagents. Plasma was serially diluted threefold from 1:9 for sTNFR1, 1:200 for sCD14 and 1:3 for sBAFF enzyme linked immunosorbent assay (ELISA). Binding of the peroxidase-conjugated secondary antibody was detected with a tetramethylbenzidine (TMB) substrate (Sigma-Aldrich; St Louis, MI, USA). Reactions were stopped with 1 M sulfuric acid (H2SO4) and quantified at 450 nm. Four-parameter logistic curves were generated from >6 titrations of the standard using SOFT max PRO version 5.4 software.

Total IgG was quantified using plates coated with polyvalent goat anti-human IgG (2.5 mg/mL; Invitrogen; Carlsbad, CA, USA) diluted 1:500 in bicarbonate buffer and blocked with 5 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 60 min. Plasma samples were serially diluted threefold from 1:1000, in 2 % BSA/PBS and applied for 120 min. Binding was detected using goat anti-human IgG conjugated horseradish peroxidase (HRP) (Sigma-Aldrich) followed by TMB substrate as described above. Hypergammaglobulinemia was defined as >14 g/L IgG, as the normal range is 6-14 mg/mL [33].

CMV-specific antibody ELISAs
IgG reactive with CMV was quantitated using CMV lysate, CMV gB and CMV IE1. CMV lysate was prepared by sonication of human foreskin fibroblasts (HFF) infected with CMV strain AD169. Uninfected HFF were prepared in parallel to create a control lysate. Replicate plates were coated with CMV gB (produced in hamster ovary cells, Chiron Diagnostics, Medfield, MA, USA; 2800–800; 50 ng/mL) or CMV IE1 (produced in E.coli, Miltenyi Auburn, CA; 2500 ng/mL). Plasma samples were diluted from 1:200. Bound IgG was detected as described above. A plasma sample assigned a value of 1000 units IgG reactive with each antigen was run on each plate, and unit values were derived for all samples. The units calculated from uninfected fibroblasts were subtracted from those generated using CMV-coated plates.

Detection of IFN-γ producing T-cells
ELISpot assays utilised anti-IFN-γ antibodies purchased from MabTech (Stockholm, Sweden) and cryopreserved PBMC with cell viability >95 % described elsewhere [26]. Cells were stimulated with anti-CD3 (10 ng/mL; MabTech), whole CMV lysate (described above), CEF control peptide pool [contains peptides from CMV, Epstein-Barr virus (EBV) and Influenza; 2 μg/mL; MabTech], CMV pp65 peptide pool (NIH AIDS reagent program, DAIDS, NIAID and Immunodiagnostics, Woburn, MA, USA), HLA-A*02-restricted peptides NLV (residues 495–503 of pp65) or VLE (residues 316–324 of IE-1) at 10 μg/mL (Proteomics International, Perth, Australia). Spots >10 units in size and >20 units in intensity were counted using an AID ELISpot Reader System (AID, Strassberg, Germany). Numbers of spots in unstimulated wells were subtracted...
from numbers in stimulated wells and adjusted per $2 \times 10^6$ PBMC.

Statistical analysis
Results were presented as median (range) values unless otherwise stated. Bivariate analyses were based on Mann Whitney tests. Correlation coefficients were determined by the Spearman’s Rank Correlation Test (GraphPad Prism version 5, La Jolla, CA, USA). For all tests, $P < 0.05$ were considered to represent a significant difference and highlighted in bold, whereas $0.05 < P < 1$ is italicized.

Additional file

Additional file 1: Figure S1. Senescent T cell gating strategy. Lymphocytes were distinguished from monocytes by their forward and side light scatter (a), gated for expression of CD3 (b), CD4 and CD8 (c). Quadrant gates were then set for expression of CD45RA and CD27 within the CD4$^+$ (d) and CD8$^+$ (e) populations. Gating was further set for expression of CD57$^+$ within the CD45RA$^-$ CD27$^-$ CD4$^+$ (f) and CD45RA$^+$ CD27$^-$ CD8$^+$ (g) populations. (PDF 261 kb)

Abbreviations
AIDS: Acquired immune deficiency syndrome; ART: Antiretroviral therapy; AU: Arbitrary units; BSA: Bovine serum albumin; CD-: Cluster of differentiation; CEF: CMV, EBV and influenza control peptide pool; CMV+: CMV seropositive healthy controls; CMV-: CMV seronegative healthy controls; CMV: Cytomegalovirus; CMV IE1: Immediate early 1 protein; CPE: Cytolytic activity of pp65 expressing CMV-infected CEF cells; CPM: Count per minute; CMV pp65: Cytomegalovirus pp65; CTV: Cytotoxic T lymphocytes; DNA: Deoxyribonucleic acid; EBV: Epstein-Barr virus; ELISA: Enzyme linked immunosorbent assay; ELISPOT: Enzyme linked immunospot assay; FBS: Fetal bovine serum; FCM: Flow cytometry; FMO: Fluorescence minus one; FMOG: Fluorescence minus one, guest; FMOC: Fluorescence minus one, control; FMOC: Fluorescence minus one, control; FMOG: Fluorescence minus one, guest; FSC: Forward scatter; GSH: Glutathione; Hesperidin: Hesperidin; HRP: Horseradish peroxidase; IE1: Immediate early 1 protein; IFN: Interferon; IgG: Immunoglobulin G; IL: Interleukin; IgA: Immunoglobulin A; IgM: Immunoglobulin M; IL-2: Interleukin 2; IL-7: Interleukin 7; IL-12: Interleukin 12; IL-6: Interleukin 6; IL-10: Interleukin 10; IV: Intravenous; HLA: Human leukocyte antigen; HRP: Horseradish peroxidase; IE1: Immediate early 1 protein; IFN: Interferon gamma; IgG: Immunoglobulin G; NLV: NLVPMVATV peptide from CMV pp65 protein; PBMC: Peripheral blood mononuclear cells; PBS: Phosphate buffered saline; pp65: CMV structural protein; sBAFF: Soluble BAFF; TMB: Tetramethylbenzidine; TCV: T cell virology; TCR: T cell receptor; TCM: T cell memory; TCM: T cell memory; TM: Tumor marker; TNF: Tumor necrosis factor; VLE: VLEETSVML peptide from CMV IE1 protein.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JA and JM carried out the patient and healthy control recruitment and flow cytometry. JA performed the ELISpot assays. JA and SB carried out the ELISA assays. JA analysed all the data, statistical analysis and drafted the manuscript. DN participated in the design of the study and patient recruitment. PP conceived the study, participated in its design and coordinated the drafting of the manuscript. All authors read and approved the final manuscript.

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Supplementary Figure 1 – Senescent T cell gating strategy

(a) CD4 T cells
(b) CD8 T cells
(c) CD4+ CD27+ CD45RA+ cells
(d) CD27 expression
(e) CD45RA expression
(f) CD57 expression
(g) CD45RA and CD57 expression
Chapter 6a

HIV patients stable on ART retain evidence of a high CMV load but changes to Natural Killer cell phenotypes reflect both HIV and CMV.


This chapter is presented in the form accepted for publication.

*Chapter outline:*

Chapter 6a focuses on investigating whether phenotypic profiles of NK cells in HIV patients stable on ART remain abnormal and reflect the immunological “footprint” of CMV.
HIV patients stable on ART retain evidence of a high CMV load but changes to Natural Killer cell phenotypes reflect both HIV and CMV

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Short Title: CMV and HIV differentially affect NK cells
ABSTRACT

Background: Whilst ART corrects many effects of HIV disease, T-cell populations retain features of accelerated immunological aging. Methods: Here we analyse phenotypic changes to natural killer (NK) cells in HIV patients who began ART with <200 CD4 T-cells/µl and maintained virological control for 12-17 years, compared with CMV seropositive and seronegative healthy control donors. Results: Humoral responses to CMV antigens (lysate, gB, IE-1) remain elevated in the patients (P<0.0001) despite the long duration of ART. Patient’s NK cells responded poorly to K562 cells when assessed by CD107a and IFNγ, but this could not be attributed to CMV as responses were low in CMV-seronegative controls. Moreover HIV (and not CMV) increased expression of CD57 on CD56lo cells. Conclusions: Comparisons with published studies suggest that CMV accelerates age-related increases in CD57 expression but levels plateau by 60-70 years of age, so the effect of CMV disappears. In HIV patients the plateau is higher and perhaps reached sooner.

150 words

Key words: antiretroviral therapy, CD57, CMV, HIV, natural killer cells
INTRODUCTION

Over 50% of healthy individuals and 90% of individuals living with HIV are seropositive for cytomegalovirus (CMV). Retinitis is the most common manifestation of CMV disease in human immunodeficiency virus -1 (HIV) -infected individuals, affecting up to 40% of American acquired immune deficiency syndrome (AIDS) patients 1, and 5-25% of HIV patients in the developing world 2. As CMV prophylaxis is expensive, it is suspended once patients are stable on antiretroviral therapy (ART). However CMV establishes latency with frequent reactivation triggered by inflammatory mediators, including tumour necrosis factor (TNF) 3. Immune activation persists in HIV patients responding to ART 4 and levels of TNFα remain elevated [e.g. 5], so subclinical reactivations of CMV triggered by these factors may continue on ART 3. Indeed titres of antibody reactive with CMV increase in the first year on ART. They decline thereafter but remain above those seen in age-matched healthy controls. This cannot be explained by hypergammaglobulinaemia 6 and likely reflects a high burden of CMV persisting on ART.

Associations between CMV replication and cardiovascular disease include studies of clinical material linking atherosclerosis with induction of adhesion molecules in endothelial tissues actively infected with CMV 7. This fits clinical evidence associating increased T-cell responses to CMV with more severe cardiovascular changes in HIV patients 8. In addition, CMV drives T-cells to replicative senescence. In older CMV-seropositive adults (not infected with HIV), up to 23% of the T-cell population can be CMV-specific 9,10. CMV-specific T-cells carry an immunosenescent phenotype that includes expression of CD57 and limited proliferative potential. T-cell senescence can impair T-cell homeostasis when thymic activity is depressed, as it is in HIV patients. Indeed T-cell expression of CD57 and an associated switch from interleukin (IL)-2 to interferon gamma (IFNγ) production are evident in HIV patients with a stable response to ART 11.

Several lines of evidence suggest that natural killer (NK) cells are important in the control of CMV. Direct evidence is provided by a congenitally T-cell deficient child whose acute CMV infection was accompanied by an expansion of NK cells which resolved with her
plasma CMV-deoxyribonucleic acid (DNA) 12. Teleological evidence also implicates NK cells. Human and mouse CMV diverged with their host species during mammalian evolution but both encode host proteins able to subvert NK-mediated killing 13. In HIV patients, NK cells also retain the imprint of the pre-ART immune system for many years. For example after 8 years on ART, IFNγ responses of CD4 T-cells to CMV were inversely related to CD4 T-cell counts before ART in patients who began ART with <60 CD4 T-cells/μL, but IFNγ responses of NK cells to a target cell lacking ligands for inhibitory receptors (K562 cells) were directly proportional to nadir CD4 T-cell counts 14. NK cell IFNγ responses remained lower than in controls, confirming persistent NK dysfunction 15.

Here we address whether phenotypic profiles of NK cells in HIV patients stable on ART remain abnormal and reflect the immunological “footprint” of CMV. For this purpose, the lifetime burden of CMV is estimated from the levels of antibodies reactive with CMV lysate, glycoprotein B (gB) protein or immediate early (IE)-1 antigen, or CD4 T-cell IFNγ responses.

Leukocyte immunoglobulin-like receptor -1 [LIR-1 (ILT2, LIRB1, CD85j)] expression was proposed as an early marker of CMV replication in transplant recipients, as its expression is increased before CMV-DNA appeared in plasma 16,17. Increased expression of LIR-1 on NK cells was associated with atherosclerosis 18, consistent with a role for CMV in atherogenesis. CD57 expression by NK cells marks terminal differentiation. CD57+CD56loNKG2C+ NK cells have poor proliferative capacity and are enriched in organ transplant patients with active CMV infections 19,20. Our study describes HIV patients who began ART with <200 CD4 T-cells/μl and maintained virological control for 12-17 years. We selected this narrow demographic band as it remains the best long-term outcome for patients beginning ART with advanced HIV disease. It is pertinent to establish the benefits that they may accrue from CMV therapy.
RESULTS

**Humoral responses to all CMV antigens remain elevated in HIV patients stable on ART**

All HIV patients had begun ART with low CD4 T-cell counts and achieved significant immune reconstitution [median (interquartile range): nadir 42 (23-142) CD4 T-cells/µl and current 691 (576-889) CD4 T-cells/µl after 174 (165-185) months on ART]. HIV patients and CMV seropositive (CMV+) healthy controls were similar in age, but the CMV seronegative (CMV-) controls were a little younger (Table 1).

Levels of antibodies reactive with CMV lysate, CMV gB and CMV IE1 were higher in HIV patients than CMV+ controls. In HIV patients, levels of antibodies reactive with CMV antigens were tightly correlated (R=0.57 to 0.81, P=0.009 to <0.0001). The relationship was similar in CMV+ controls (R=0.52 to 0.57, P=0.02 to 0.18) and levels of antibody reactive with lysate and gB were correlated in CMV- controls (R=0.75, P=0.025), consistent with everyone having some exposure to CMV in their lifetime. Antibody levels were not influenced by age, perhaps because the age range was limited (50 to 73 years). Antibody levels in HIV patients were steady or declined marginally on ART (R= -0.34 to -0.05, P = 0.14 to 0.85).

Compared with the clear difference in humoral responses to CMV, CD4 T-cell IFN\(\gamma\) responses to CMV lysate did not differ between HIV+ CMV+ patients and CMV+ controls (P=0.16). However IFN\(\gamma\) and antibody responses to CMV lysate were suggestive of association in CMV+ controls (R=0.44, P=0.08,) but not in patients (R=0.25, P=0.29). From these comparisons, we selected antibody titres rather than IFN\(\gamma\) responses to assess the footprint of CMV on NK cells in HIV patients.
NK cell function is depressed by stably treated HIV disease, without the boost to NK function attributed to CMV in controls

HIV patients had higher proportions of CD8 T-cells and slightly lower proportions of NK cells than CMV+ or CMV- controls individually (Table 1), with significantly less NK cells than the combined control groups ($P=0.03$). Whilst the proportions of CD56$^{lo}$ and CD56$^{hi}$ cells did not differ between groups, the CD56$^{hi}$/CD56$^{lo}$ ratio was slightly higher in CMV-controls ($p=0.01$ and $p=0.06$ vs HIV patients and CMV+ control resp.). These differences are not considered clinically informative.

NK cell function was then assessed from the proportion of NK cells expressing CD107a or IFN$\gamma$ with and without stimulation with K562 cells. These markers were predominantly expressed on CD56$^{lo}$ NK cells (See Supplementary Figure 1) and their expression was invariably increased by culture with K562 cells. After stimulation with K562 cells, expression of CD107a and IFN$\gamma$ by NK cells was lower in HIV patients than in CMV+ controls (Table 1). Moreover NK IFN$\gamma$ responses were lower in HIV patients who had been on ART for longer periods ($R = -0.60$, $P=0.01$), with CD107a following a similar trend ($R = -0.34$, $P=0.15$). IFN$\gamma$ production gated specifically to CD56$^{hi}$ NK cells was not increased by K562 cells in any group ($P=0.46$ to 0.96; data not shown), but followed a pattern similar to that seen with CD56$^{lo}$ NK cells. Specifically, expression in unstimulated cultures from HIV patients was slightly lower than from CMV+ controls and was inversely proportional to time on ART ($R= -0.41$, $P=0.09$).

CD107a responses were lowest in CMV- controls, so the low NK responses of HIV patients occur despite their greater humoral responses to CMV. Indeed HIV patients displayed variable but always negative correlations between CD107a expression (+/− K562 cells) and CMV antibody (lysate, gB, IE-1; $R = -0.11$ to -0.48). IFN$\gamma$ production by CD56$^{lo}$ or CD56$^{hi}$ NK cells did not correlate consistently with levels of CMV antibody in any group of donors ($R= -0.58$ to 0.33).
**LIR-1 expression was not related to levels of CMV antibody, but associated with CD107a responses in HIV patients**

The expression of LIR-1 on NK cells and CD8 T-cells was similar in CMV+ HIV patients and control donors, and slightly lower in CMV- controls. This trend was evident when LIR-1+ cells were assessed as a percentage of NK cells or CD8 T-cells (data not shown) or by the median fluorescent intensity (MFI) of LIR-1 (Table 1; Supplementary Figure 1), but did not reach statistical significance. Levels of antibody reactive with CMV did not correlate with the MFI of LIR-1 on NK cells or CD8 T-cells in HIV patients (R= -0.38 to 0.06) or CMV+ (R= -0.17 to 0.49) or CMV- (R= -0.40 to -0.10) controls.

LIR-1 is an inhibitory receptor but its ligand [human leukocyte antigen-G (HLA-G)] is not found on K562 cells (21), so correlations between LIR-1 expression and NK responses to this target are indirect. In HIV patients, NK expression of CD107a correlated with expression of LIR-1 on NK cells (Table 2) and CD8 T-cells (P=0.05, R=0.45 unstimulated; P=0.01, R=0.55 with K562). CD107a responses did not correlate with expression of LIR-1 on NK cells (Table 2) or CD8 T-cells (P > 0.10; R = 0.20 to 0.49) in CMV+ or CMV- controls, but this may reflect the smaller sample sizes. Correlations were also clearer in patients than CMV+ or CMV- controls when LIR-1+ cells were gated (data not shown).

**CD57 expression was not related to levels of CMV antibody, but was increased and correlated with CD107a and perforin responses of CD56lo NK cells in HIV patients**

CD57 was expressed on a higher proportion of NK cells from HIV patients than CMV+ controls (P=0.02). Expression was similar in CMV+ and CMV- controls, and was not proportional to levels of CMV antibody in HIV patients (P = 0.22 to 0.57, R = -0.13 to -0.29). Examination of the flow cytometry plots confirmed that CD57 was expressed on CD56lo NK cells in all donors (Supplementary Figure 1; 19).

In HIV patients, CD57 expression on NK cells correlated directly with expression of CD107a, with a weak inverse association with IFNγ (+/- K562 cells). HIV patients also demonstrated a striking correlation between CD57 expression on NK cells and the CD56loPerforinhi phenotype. This was complemented by an inverse correlation between
CD57 and the CD56hiPerforinlo phenotype (Table 2). No associations were seen in control donors.

DISCUSSION

The effects of HIV disease and ART on NK phenotypes are unclear. Azzoni et al\(^2\) describe low NK cell numbers and poor NK cell cytotoxicity during untreated HIV infection, with limited recovery over 1-2 years on ART. Goodier et al\(^2\) reported that NK IFN\(\gamma\) responses remain low on ART despite \textit{in vitro} stimulation with IL-2, IL-12 and IL-15. However Alter et al\(^2\) demonstrated increased IFN\(\gamma\) and CD107a responses per NK cell in viremic patients with resolution over one year on ART. The contribution of CMV to changes attributed to HIV may be critical and is addressed here.

Our study describes patients who began ART with <200 CD4 T-cells/\(\mu\)l and maintained virological control for 12-17 years. We confirmed that humoral responses to CMV antigens (lysate, gB, IE-1) remain elevated above levels seen in CMV-seropositive controls (\(P<0.0001\)) and linked this with T-cell senescence and low nadir CD4 T-cell counts \(^2\). Patients had slightly lower proportions of NK cells but their ratios of CD56hi/CD56lo cells were similar to controls.

We then assessed the functional capacity of NK cells using IFN\(\gamma\) and expression of CD107a by NK cells. IFN\(\gamma\) is produced when NK cells become activated \(^2\), whereas CD107a lines the membrane of cytolytic granules and is detected when the granule fuses with the cell membrane \(^2\). HIV patients responded poorly to K562 cells when assessed by CD107a and IFN\(\gamma\) on CD57lo NK cells, but this could not be attributed to their burden of CMV as responses were lowest in CMV-seronegative controls. In contrast, HIV patients described by Lima et al\(^2\) displayed normal to elevated NK responses to phorbol myristate acetate (PMA)/calcium (Ca) ionophore. This may reflect the means of stimulation, as PMA/Ca ionophore bypasses the NKG2D-mediated mechanisms invoked by K562 cells \(^2\).
LIR-1 has been proposed as a measure of the "footprint" of CMV as its expression is increased on NK cells from CMV-seropositive individuals\textsuperscript{16-18}. LIR-1 expression on NK cells has not been monitored during HIV infection but LIR-1 is implicated in the control of HIV replication\textsuperscript{29}. Here expression of LIR-1 was marginally lower in CMV seronegative controls, but was not affected by HIV or proportional to levels of CMV antibody in any group. Whilst this precludes its use as a measure of the CMV footprint in this setting, we noted a relationship between LIR-1 and CD107a expression in HIV patients. This probably means that LIR-1 expression is a property of NK cells that are healthy enough to kill, as LIR-1 is an inhibitory receptor and is not implicated in the killing of K562 cells since they don’t express HLA-G\textsuperscript{28}.

CD57 has been proposed as a marker of "memory" NK cells that have been expanded in response to CMV, as NK cells co-expressing the activating NKG2C receptor and CD57 are expanded by CMV seropositivity in healthy college students, but not by active infection with Epstein-Barr virus (EBV)\textsuperscript{30}. Here HIV (and not CMV) increased the expression of CD57 on CD56\textsuperscript{lo} cells. This was also seen in a mixed cohort of viraemic and aviraemic HIV patients (n=13), where patients displayed 40% CD57\textsuperscript{+} NK cells vs 20% in controls. CD57 expression correlated with levels of CMV antibodies in each group\textsuperscript{30}, which was not evident in our study. However the patients and controls had a broad range of ages (25-66 years). A recent study\textsuperscript{31} showed expression of CD57 increased with age from \textasciitilde50% of CD56\textsuperscript{lo} NK cells in CMV+ donors aged under 35 years to 65-70% in those \textasciitilde70 years old. CMV-seronegative younger donors had just 30-35% CD57\textsuperscript{+} NK cells. This is consistent with our data as our control donors (CMV+ and CMV-) were 50-70 years old with \textasciitilde64% CD57\textsuperscript{+} NK cells. It makes our finding that 77(59-83)% of NK cells from HIV patients express CD57 all the more striking. We conclude that CMV accelerates age-related increases in CD57 expression but levels plateau by 60-70 years of age. In HIV patients the plateau is higher and perhaps reached sooner.
Overall, the effects of HIV disease on NK cells in patients stably controlled on ART were most clearly marked by expression of CD57. Elevated expression of CD57 on NK cells from HIV patients is likely to have functional consequences as it correlated directly with CD107a and perforin by CD56lo cells and inversely with IFNγ responses and the CD56hi perforinlo phenotype. In this older and stably treated patient cohort, CD57 expression was not proportional to the burden of CMV as measured by levels of antibody. The inclusion of rare CMV-seronegative HIV patients and parallel sensitive assessments of CMV DNA are needed to define the roles of CMV and HIV in NK dysfunction.

METHODS AND MATERIALS

Patients and controls

Twenty CMV-seropositive HIV patients were recruited at Royal Perth Hospital, Western Australia, on the basis of being >50 years old with nadir CD4 T-cell counts <200 cells/µl, with >10 years on ART and >1 year of complete viral suppression (<50 HIV RNA copies/ml). ART comprised at least three antiretroviral drugs. Sixteen CMV-seropositive healthy controls (CMV+) and nine male CMV-seronegative healthy controls (CMV-) were included. All donors were Caucasian. CMV seropositivity in patients and controls was defined as >1100 AU/mL CMV lysate antibody, where the cut-off was based on eleven samples from persons negative by routine serology and detection of CMV DNA (Abbott, Lake Forrest, IL). Informed consent was obtained from all participants in accordance with approvals from Royal Perth Hospital.

Sample collection and storage

Peripheral blood mononuclear cells (PBMC) were isolated by ficoll density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare Biosciences, Sweden). 2.0 x 10⁶ cells were set aside for flow cytometry, whilst remaining cells were resuspended at 10 x 10⁶ cells/mL in fetal calf serum with 10% dimethyl sulfoxide (DMSO) for storage in liquid nitrogen.
**CMV antibody and IFNγ responses**

IgG reactive with CMV was quantitated using CMV lysate, CMV gB and CMV IE-1 antigens. CMV lysate was prepared by sonication of human foreskin fibroblasts (HFF) infected with CMV strain AD169. Uninfected HFF were prepared and extracts were run in parallel. Replicate ELISA plates were coated with CMV gB (2800-800 produced in hamster ovary cells, Chiron Diagnostics, Medfield, MA; 50ng/mL) and CMV IE-1 (130-092-137, produced in *E.coli*; Miltenyi Biotech, Bergisch Gladbach, Germany; 2500ng/mL). Plasma samples were diluted from 1:200. Binding was detected using goat anti-human IgG conjugated horse radish peroxidase (HRP), followed by tetramethylbenzidine (TMB) substrate (Sigma-Aldrich; St Louis, MI, USA). Four-parameter logistic standard curves were generated from titrations of a plasma sample assigned a value of 1100 arbitrary units (AU) IgG reactive with each and unit values were derived for all samples. Units of antibody detected on plates coated with uninfected HFF were subtracted from those generated using CMV-coated plates. IFNγ responses of CD4 T-cells were assessed by ELISpot assay as described previously.  

**NK cell and T cell phenotypes assessed ex vivo by flow cytometry**

Fresh PBMC (0.5 x 10⁶ cell per tube) were resuspended in 20µl phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) and antibodies were added for 15mins (room temperature). This included PE-ILT2 (LIR-1) clone GHI/75 (Biolegend San Diego, CA), APC-CD57 clone NK-1, PerCP-Cy5.5-CD3 clone SK7, V450-CD56 clone B159, V500-CD8 clone RPA T8 (BD Biosciences, San Jose, CA). 250,000 events were acquired using a FACS Canto II cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, San Carlos, CA). Gating strategies are illustrated in Supplementary Figure 1.
**Assessment of NK function**

K562 cells were propagated in RPMI/10% FCS and sub-cultured 24 hours before use. Cryopreserved PBMC were washed in RPMI and cultured in RPMI/5% fetal calf serum (FCS) at 1 x 10^6 cells/mL with K562 cells at a target:effector ratio of 10:1. After 6 hours (37°C), cells were transferred to flow tubes in PBS/1%BSA, washed and 10µL FcR Block (Miltenyi Biotech) was added for 20 minutes (4°C). Cells were stained for extracellular markers [PerCP-Cy5.5-CD3, V450-CD56, V500-CD8, APC-CD107a clone H4A3] (BD Biosciences) for 15 minutes (room temperature). They were then washed, 250µL Cytofix/Cytoperm (BD Biosciences) was added for 20 minutes (4°C), followed by antibodies reactive with intracellular markers [FITC-perforin clone γG9; PE-INFγ clone 4S.B3] (BD Biosciences) for 30 minutes. Washed cells were resuspended in PBS/1% BSA and 500,000 events were acquired and analyzed (See Supplementary Figure 1).

**Statistical analysis**

Results were presented as median (interquartile range) unless otherwise stated. Bivariate analyses were based on Mann Whitney tests or Fisher’s test as indicated in the Table 1. Correlation coefficients were determined by the Spearman’s Rank Correlation Test (GraphPad Prism version 5 for Windows, GraphPad Software, La Jolla, CA). For all tests, $P < 0.05$ was considered to represent a significant difference and highlighted in **bold**, whereas $0.05 < P < 1$ is *italicized.*
LIST OF ABBREVIATIONS

AIDS  Acquired immunodeficiency syndrome
ART  Antiretroviral therapy
AU  Arbitrary units
BSA  Bovine serum albumin
Ca  Calcium
CD-  Cluster of differentiation
CMV-  CMV seronegative
CMV  Cytomegalovirus
CMV+  CMV seropositive
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
EBV  Epstein-Barr virus
ELISA  Enzyme linked immunosorbent assay
ELISpot  Enzyme linked immunosorbent spot assay
FCS  Fetal calf serum
gB  Glycoprotein B
HFF  Human foreskin fibroblasts
HIV-1  Human immunodeficiency virus-1
HLA-  Human leukocyte antigen
HRP  Horseradish peroxidase
IE1  Immediate early 1 protein
IFN\(\gamma\)  Interferon gamma
IgG  Immunoglobulin G
IL-  Interleukin-
LIR-  Leukocyte immunoglobulin-like receptor-
MFI  Mean fluorescence intensity
NK  Natural killer cells
NKG2D  Natural-killer group 2, member D
PBMC  Peripheral blood mononuclear cells
PBS  Phosphate buffered saline
PMA  Phorbol myristate acetate
RNA  Ribonucleic acid
RPMI  Roswell Park Memorial Institute medium
TMB  Tetramethylbenzidine
TNF  Tumour necrosis factor
COMPETING INTERESTS
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
JA and JM carried out the patient and healthy control recruitment and flow cytometry. JA carried out the CMV lysate antibody ELISA assay. JA and PP analysed all the data and statistical analysis. PP conceived the study, participated in its design and coordinated the drafting of the manuscript. All authors read and approved the final manuscript.

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REFERENCES


Supplementary Figure 1:

The figure summarises the gating strategy used to define NK cell subpopulations, illustrated using typical control donors.
Table 1: HIV patients stable on ART retain high titres of CMV reactive antibodies, with NK cell phenotypes and function affected by HIV and CMV status.

<table>
<thead>
<tr>
<th></th>
<th>HIV patients A</th>
<th>CMV + Controls B</th>
<th>CMV- Controls C</th>
<th>A vs B</th>
<th>A vs C</th>
<th>B vs C</th>
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<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>16</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male:Female</td>
<td>19:1</td>
<td>14:2</td>
<td>9:0</td>
<td>0.57c</td>
<td>1</td>
<td>0.52</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62 (57-68)</td>
<td>60 (57-62)</td>
<td>55 (53-59)</td>
<td>0.19</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>CMV lysate IgG (AU/ml)</td>
<td>94 (56-240)</td>
<td>20 (10-35)</td>
<td>0.9 (0.6-1.0)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CMV gB IgG (AU/ml)</td>
<td>127(90-172)</td>
<td>45 (27-55)</td>
<td>1.9 (1.1-2.8)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>CMV IE-1 IgG (AU/ml)</td>
<td>49 (21-166)</td>
<td>8 (4.3-180)</td>
<td>2.6 (2.0-3.7)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0017</td>
</tr>
<tr>
<td>CMV lysate IFNγ e</td>
<td>227(16-700)</td>
<td>157 (13-617)</td>
<td>0 (0-0.5)</td>
<td>0.16</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>NK cells (% lymphocytes)</td>
<td>12(8.3-18)</td>
<td>17(11-20)</td>
<td>17(12-22)</td>
<td>0.09</td>
<td>0.07</td>
<td>0.76</td>
</tr>
<tr>
<td>CD8 T-cells (% lymphocytes)</td>
<td>39(19-48)</td>
<td>17(7-23)</td>
<td>13(10-19)</td>
<td>0.0007</td>
<td>0.001</td>
<td>0.71</td>
</tr>
<tr>
<td>CD56hi/CD56lo ratio*100</td>
<td>7.2 (3.5-18)</td>
<td>7 (2.5-9.8)</td>
<td>12 (9.0-26)</td>
<td>0.51</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>IFNγ+ (%NK cells) d</td>
<td>0.2 (0.1-0.32)</td>
<td>0.1 (0.1-0.38)</td>
<td>0.4 (0.15-0.55)</td>
<td>0.93</td>
<td>0.19</td>
<td>0.17</td>
</tr>
<tr>
<td>IFNγ+ (%NK cells) + K562</td>
<td>0.8 (0.3-1.10)</td>
<td>1.7 (1.0-2.6)</td>
<td>1 (0.65-2.75)</td>
<td>0.006</td>
<td>0.21</td>
<td>0.4</td>
</tr>
<tr>
<td>CD56hiIFNγlo (%NK cells) d</td>
<td>0.36 (0.07-1.3)</td>
<td>1.1 (0.48-1.6)</td>
<td>0.4 (0.16-0.97)</td>
<td>0.08</td>
<td>0.96</td>
<td>0.12</td>
</tr>
<tr>
<td>CD107a+ (%NK cells) d</td>
<td>6.3 (2.6-8.6)</td>
<td>6.2 (4.6-7.9)</td>
<td>3.0 (2.2-6.0)</td>
<td>0.86</td>
<td>0.16</td>
<td>0.03</td>
</tr>
<tr>
<td>CD107a+ (%NK cells) + K562</td>
<td>11 (6.3-14)</td>
<td>16 (9.8-17)</td>
<td>8.4 (6.1-11)</td>
<td>0.03</td>
<td>0.48</td>
<td>0.006</td>
</tr>
<tr>
<td>LIR-1 MFI (NK cells)</td>
<td>554 (419-706)</td>
<td>524 (351-774)</td>
<td>301 (270-734)</td>
<td>0.82</td>
<td>0.27</td>
<td>0.17</td>
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<tr>
<td>LIR-1 MFI (CD8 T cells)</td>
<td>494 (385-679)</td>
<td>526 (429-852)</td>
<td>374 (283-807)</td>
<td>0.3</td>
<td>0.07</td>
<td>0.15</td>
</tr>
<tr>
<td>CD57+ (%NK cells)</td>
<td>77 (59-83)</td>
<td>64 (54-66)</td>
<td>63 (59-65)</td>
<td>0.02</td>
<td>0.09</td>
<td>0.98</td>
</tr>
<tr>
<td>CD56lo perf hi (%NK cells) d</td>
<td>85 (76-90)</td>
<td>83 (77-89)</td>
<td>79 (70-87)</td>
<td>0.77</td>
<td>0.29</td>
<td>0.25</td>
</tr>
<tr>
<td>CD56hi perflo (%NK cells) d</td>
<td>3.0 (1.0-5.9)</td>
<td>3.1 (1.7-5.3)</td>
<td>5.2 (2.9-5.3)</td>
<td>0.88</td>
<td>0.07</td>
<td>0.08</td>
</tr>
</tbody>
</table>

a Median (interquartile range)  
b Mann Whitney tests (continuous data), P < 0.05 (bold), P > 0.05-0.1 (italics)  
c Fisher’s exact test (categorical data)  
d cultured without K562 cells  
e IFNγ spots per 2 x 10⁶ cells
Table 2: In HIV patients, NK expression of CD57 correlated directly with CD107a⁺ and perforin⁺ expression on CD56lo cells and inversely with IFNγ responses and the CD56hi perforinlo phenotype

<table>
<thead>
<tr>
<th>Population assessed as % NK cells expressing...</th>
<th>HIV patients</th>
<th>CMV + Controls</th>
<th>CMV- Controls</th>
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<tr>
<td>LIR-1 (MFI on NK cells) vs....</td>
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<tr>
<td>CD107a</td>
<td>0.01</td>
<td>0.15</td>
<td>0.49</td>
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<tr>
<td>CD107a with K562</td>
<td>0.02</td>
<td>0.26</td>
<td>0.49</td>
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<td>CD57 vs....</td>
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<tr>
<td>CD107a</td>
<td>0.02</td>
<td>0.73</td>
<td>0.43</td>
</tr>
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<td>CD107a with K562</td>
<td>0.08</td>
<td>0.28</td>
<td>-0.30</td>
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<tr>
<td>IFNγ</td>
<td>0.10</td>
<td>-0.41</td>
<td>0.61</td>
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<tr>
<td>IFNγ with K562</td>
<td>0.14</td>
<td>-0.35</td>
<td>0.17</td>
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<tr>
<td>CD56lo perforinhi</td>
<td>0.001</td>
<td>0.28</td>
<td>0.49</td>
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<tr>
<td>CD56hi perforinlo</td>
<td>0.004</td>
<td>0.86</td>
<td>0.64</td>
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a Spearman’s correlation test.
Supplementary Figure 1: The figure summarises the gating strategy used to define NK cell subpopulations, illustrated using typical control donors.
Chapter 7

General Discussion
CHAPTER 7 - GENERAL DISCUSSION

Genetic and immunological factors are often implicated in the outcome and prognosis of chronic infectious diseases. A better understanding of the genetic risk factors that determine host susceptibility could provide important leads for improved therapies. To identify useful disease-related biomarkers, the first part of my thesis investigated the effects of SNPs affecting cytokines, chemokines and their receptors, on susceptibility to clinical exacerbations. Carriage of TNFA-1031, SLC11A1 D543N or IL10-1082 were the most promising genetic “markers” predisposing to TB-IRD, NTM or CMV diseases. As low nadir CD4 T cell counts is a hallmark of CMV disease in HIV patients, elevated levels of B cell activating factor may be another useful indicator of an immunological “footprint” implicated in accelerated immune ageing.

The following chronic infectious diseases were studied in the context of HIV, a condition driven by viral replication in CD4 T cells – TB-IRD (Chapter 3) and CMV (Chapter 5). TB-IRD was compared with NTM disease (Chapter 4), a condition clinically and radiographically indistinguishable from TB. SNPs associated with susceptibility to increased risk of disease will be discussed here in Section 1. A summary of the genetic associations identified in Chapters 3, 4 and 5 is presented in Table 1. In the second part of my thesis, I investigated immunological factors associated with the long-term effects of CMV on the host immune system via its effects on T-cell senescence. In immunocompetent healthy individuals, CMV infection is usually asymptomatic or mildly symptomatic, however it can lead to morbidity and mortality in HIV patients. Affronts to the immune system potentiates CMV reactivation and thus immune activation persists leading to immunosenescence. Understanding the mechanisms behind these effects is the basis of my study which will be discussed in section 2.
Table 1: Summary of genetic associations identified in Chapters 3, 4 and 5

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Disease</th>
<th>[wild type/variant] a</th>
<th>rs number</th>
<th>Carriage of allele</th>
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<td>TB-IRD</td>
<td>Cambodia</td>
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<td></td>
<td></td>
<td>TNFA-1031 [T/C]</td>
<td>1799964</td>
<td>C</td>
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<td></td>
<td></td>
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a Univariate analysis only
b Multivariable modelling by logistic regression

 Bootstrap multivariable modelling by negative binomial regression
1 GENETIC ASSOCIATIONS WITH SUSCEPTIBILITY TO DISEASE

1.1 Carriage of the variant allele of TNFA-1031 associates with susceptibility to chronic infectious diseases

I demonstrated that carriage of the variant allele of TNFA-1031 was associated with susceptibility to NTM disease in Australian Caucasian patients (Chapter 4), development of IRD event in Cambodian HIV-TB co-infected patients (Chapter 3), and low nadir CD4 T-cell counts in American Caucasian HIV-CMV co-infected patients (Chapter 5).

TNFA-1031 is a polymorphism in the promoter of the TNFA region of the major histocompatibility complex (MHC) Class III on chromosome 6p21,31 (See Figure 1). This region of the MHC is highly conserved 2, and has been associated across a range of pathologies and ethnicities including, HIV-sensory neuropathy in Australian Caucasian patients 3 and Indonesian patients 4, Behcet’s disease 5,6, and polycystic ovary syndrome 7. TNF plays a prominent role in controlling mycobacterial spread and growth via macrophage activation, granuloma formation and orchestrating the cellular immune response. This cytokine and associated inflammation have been implicated in autoimmune and infectious diseases.

Levels of TNF production vary within any population, with high (GA and AA) and low producer phenotypes (GG) observed for the TNFA-308 polymorphism 8,9. This indicates a potential genetic impact on TNF regulation 1. However the impact of TNF-1031 on TNFα production has not been addressed extensively. Gupta et al reported that carriage of TNFA-1031*C increased levels of TNFα 10 and associated with risk of diabetic nephropathy. Also, investigations in Sjogren’s patients carrying TNFA-1031*C was associated with increased circulating levels of TNFα 11. TNFA-1031*CC conferred protection against leprosy development 12, and with Behcet’s disease in Moroccan patients 13. However, TNFA-1031 was not associated with susceptibility to asthma of in a Chinese study 14, or peptic ulcer or Helicobacter pylori infection in a Polish cohort 15.
TNFA promoter polymorphisms may act synergistically, hence their effects may be augmented when they are assessed as haplotypes. My colleagues associated a TNF haplotype denoted FVa 6,7,8 incorporating TNFA-1031 with HIV-sensory neuropathy in Chinese patients\textsuperscript{16} but the haplotype effect was not seen in African patients\textsuperscript{17}. Hence future studies should include the TNFA-1031 SNP with other TNF SNPs as haplotypes, which may exert a more significant influence to diseases.

\textbf{Figure 1}: Schematic diagram illustrating the major histocompatibility complex (MHC) on chromosome 6p21,31. Included are some of the genes in the TNF region and the promoter polymorphisms. Numbering of each SNP is descending from the +1 of the transcription start site (TSS). ‘Core’ promoter refers to the region showing high evolutionary conservation and well-documented transcription on factor binding and activity. (adapted from Bayley et al, 2004\textsuperscript{1})
1.2 Carriage of the wild-type allele of SLC11A1 D543N associate with susceptibility to TB-IRD and CMV end-organ disease

Carriage of allele G in SLC11A1 D543N was associated with increased risk of IRD in the HIV-TB coinfected Cambodian patients (Chapter 3) and with CMV end-organ disease in CMV seropositive African American HIV patients (Chapter 5). SLC11A1 gene encodes for the natural resistance associated macrophage protein 1 (NRAMP1) and has been associated with tuberculosis. This gene is involved in divalent cation homeostasis in macrophages, is important in macrophage-mediated natural resistance to various intracellular pathogens and influencing antigen presentation. SLC11A1 plays an essential role in macrophage activation through regulation of TNF, MHC Class II molecules, interleukin 1β as well as antimicrobial activity and induction of nitric oxide synthase. SLC11A1 D543N has been associated with predisposition to Crohn’s disease in Greek patients, Buruli ulcers in Ghanaian patients and TB in Cambodian, Chinese, Japanese and Gambian patients. However, no associations with TB were found in Turkish, Indonesian, and Thai patients. This SNP was a risk factor for treatment failure in male Mexican tuberculosis patients. These studies were conducted in ethnically distinct populations, so the roles of haplotypes, epigenetics and geographical/environmental factors may differ between studies.

Other polymorphisms in the SLC11A1 gene region have been associated with chronic immune diseases such as multiple sclerosis, sarcoidosis and visceral leishmaniasis. rs17235416 (3’UTR) and rs3731865 (INT4) have been typed together with SLC11A1 D543N in other immune-related and infectious diseases, inferring that haplotypes may be critical and warrant further investigations with the inclusion of functional studies involving these sets of SNPs.
1.3 **Carriage of allele A of IL10-1082 associate with susceptibility to NTM disease, CMV end-organ disease and predisposing low nadir CD4 T cell counts**

In patients with NTM disease, heterozygosity at IL10-1082 conferred 67% decreased odds of developing NTM disease compared to homozygous carriage of the wild-type allele. Hence, carriage of IL10-1082*AA confers an increased risk of susceptibility to NTM disease (Chapter 4). Carriage of the GA allele in IL10-1082 was linked with susceptibility to CMV end-organ disease in American Caucasians and the IL10-1082*GG allele was associated with predisposition to low nadir CD4 T-cell count (Chapter 5). IL-10 plays a crucial role as an inhibitory factor during the production of T helper 1 (Th1) and Th2 cytokines, important in maintaining an adequate balance between inflammatory and immunopathological responses\(^\text{39}\). High levels of IL-10 can reduce IL-12 and IFN\(\gamma\) production, which is important for controlling mycobacterial infection. Thus an increase in IL-10 levels may promote mycobacterial survival in the host. In a study of Cambodian anergic TB patients, IL-10 production was elevated, suggesting that MTB potentiates IL-10 induction leading to suppression of an effective immune response\(^\text{40}\).

However, the functional significance of this SNP with reference to IL10 production in current literature remains inconsistent. Wang *et al* observed that the A allele associated with increased serum IL-10 levels in patients with Crohn’s disease from New Zealand\(^\text{41}\). In contrast, carriage of the GG and GA alleles have been associated with a “high IL-10 producer” phenotype, compared to the AA allele\(^\text{42}\). Turner *et al* quantified IL-10 levels by enzyme-linked immunosorbent assay (ELISA) from 48 culture supernatants using healthy control buffy coats\(^\text{42}\). Carriage of the G allele in IL10-1082 was associated with higher expression of IL10 mRNA and serum concentration greater than 2 pg/mL\(^\text{43}\). Contradiction between the studies correlating levels of IL-10 production and genotypes can possibly be explained by allelic distribution. For example, Suarez *et al* had a higher percentage of the A allele in IL10-1082 (62%) than the G allele (38%)\(^\text{43}\), whereas Wang *et al* observed an almost equal distribution of the A (48) and G (52%) alleles\(^\text{41}\). Hence, more investigations need to be done in larger cohorts to assess levels of IL-10 production in relation to allelic distribution in various populations.
IL10-1082 is often studied in conjunction with other promoter polymorphisms IL10-592 and IL10-819 forming haplotypes that associate with diseases such as colorectal cancer \(^{44}\) and oral cancer\(^{45}\), as well as associations with IL-10 levels\(^{46}\). IL10-592 and IL10-819 affect transcription \(^{47}\) after stimulation of PBMC with Streptococcus pneumonia. \textit{IL10} haplotypes warrant further investigation because high production of IL-10 by peripheral blood mononuclear cells (PBMC) from NTM patients has been observed \(^{48}\).

1.4 **Carriage of variant allele in IL18-607 is associated with susceptibility to TB-IRD**

In Indian HIV-TB coinfected patients, carriage of the variant allele in IL18-607 conferred a 4-fold increased incidence of IRD, but this effect was not observed in Cambodian HIV-TB patients (Chapter 3). The IL18-607 SNP was not associated with tuberculosis in HIV-negative pulmonary TB patients of Indian descent \(^{49}\) or in HIV-negative Iranian TB patients \(^{50}\). IL-18 is a pro-inflammatory cytokine that has an important role in immune defence and it has the potential to influence severe inflammatory disorders \(^{51}\). IL-18 is able to promote Th1 cell development and IFN\(_\gamma\) production, along with IL-12 or IL-15 \(^{51}\). IL18-607 transverses from C to A, disrupts a cyclic adenosine monophosphate (cAMP)-responsive element-binding (CREB) site. Haplotypes of IL-18-607 and IL18-137 can downregulate transcriptional activity \(^{52,53}\) of the gene by changing a histone 4 transcription factor-1 (H4TF-1) nuclear factor binding site \(^{54}\). A haplotype study involving IL18-607, rs5744247 and rs549908 observed that the A allele of IL18-607 conferred a 1.5 times risk of developing TB. These conflicting data may be due to differences in the linkage disequilibrium in different ethnic backgrounds.
1.5 **Carriage of variant in VDR Fok (F/f) associated with increased risk of IRD in Indian HIV-TB patients**

Polymorphisms in the vitamin D receptor (VDR) have been associated with tuberculosis and verified in diverse ethnicity and geographic locations\(^{55,56}\). Carriage of the variant allele in VDR FokI (F/f) conferred an increased risk of IRD in Indian cohort of HIV-TB coinfected patients compared to Cambodian HIV-TB patients (Chapter 3). In the appendix to Chapter 3, TB-IRD was not associated with plasma levels of Vitamin D and the VDR Fok I (F/f) allele did not predict susceptibility to an IRD event in HIV-TB coinfected patients in Cambodia, India nor South Africa\(^{57}\).

1.6 **Heterozygosity in IL28B-rs8099917 associated with susceptibility to NTM disease**

NTM disease is not easily distinguishable clinically or radiographically from TB and yet is caused by bacteria that are only weakly pathogenic, so genetic regulation of susceptibility may be particularly important. IL28B belongs to the newly discovered interferon \(\lambda\) family including IL29 and IL28A\(^{58}\). IFN\(\lambda\) structure is similar to that of IL-10\(^{59}\) and capable of modulating Th1/Th2 response\(^{60}\) and similar to type 1 interferons\(^{61}\). Heterozygosity in IL28B-rs8099917 conferred a two-fold increased risk of susceptibility to NTM disease (Chapter 4). This SNP has been extensively studied in patients infected with hepatitis C virus (HCV). Liver transplant patients carrying the TT genotype in rs8099917 responded better to IFN therapy compared with patients carrying the TG or GG genotypes\(^{62}\). Carriage of the rs8099917 TG or GG genotypes was associated with high expression of intrahepatic interferon-stimulated genes (ISGs) in HCV patients\(^{63,64}\).
1.7 **IL1A+4845*AA associated with increased risk of developing NTM disease**

In Chapter 4 of my thesis, homozygous carriage of the variant allele (A) in IL1A+4845 was associated with susceptibility to developing NTM disease in a cohort of HIV uninfected patients, compared to controls. IL1A+4845 is in linkage disequilibrium with IL1A-889. The A allele of IL1A+4845 have been implicated in respiratory disease such as the H1N1 influenza A virus 65, nasal polyposis66 and chronic rhinosinusitis 67. In previously immunodeficient HIV patients stable on ART for 5 years, variant alleles carried at IL1A-889 or IL1A+4845 were found to predict the control of HIV replication68. The IL-1 family of cytokines consists of IL-1α, IL-1β and IL-1 receptor antagonist (RA) which is a pure antagonist of former two 69 components. IL-1α and IL-1β are pleiotropic pro-inflammatory cytokines, and enhance the growth of T and B cells, as well as induce other cytokines 70. Hence, extending studies exploring polymorphisms in the *IL1* gene may elucidate aspects of the immunopathogenesis of NTM disease.

1.8 **SNPs associating with susceptibility to CMV end-organ disease and predisposition to low nadir CD4 T cell counts**

In Chapter 5 of my thesis, carriage of IL12B 3'UTR*AA in African American HIV patients, and carriage of LILRB1 I142T*TT in Caucasian American HIV patients, were associated with susceptibility to CMV end-organ disease. African Americans patients who carried the wild–type alleles of DARC T-46C and variant allele in CD14 C(-159)T, often had low nadir CD4 T cell counts. These results were not observed in the Caucasians where low nadir CD4 T cell counts associated with carriage of TNFA-308*A, TNFA-1031*TT, IL2-330*G, CCL2-2518*GG and LILRB1 I142T*C.

Associations with low nadir CD4 T-cell counts may reflect persistent immune activation potentiated by CMV seropositivity, exacerbating an already fragile immune system impaired by HIV. IL12B3’UTR SNP (also known as IL12+1188) lies within the p40 subunit encoded by *IL12B* 71, whereby IL-12 induces the production of IFNγ. This SNP has been associated with increased risk of gastric cardiac adenocarcinoma 72, rheumatoid
arthritis\textsuperscript{73} and type 2 diabetes mellitus\textsuperscript{74}. Carriage of homozygous variant allele (CC) at IL12B 3’UTR in healthy donors had significantly higher IL-12 secretion levels from lipopolysaccharides (LPS) and purified protein derivative (PPD) stimulated PBMCs than AC heterozygotes or AA homozygotes\textsuperscript{75}. Thus, if carriage of the low-producing allele A in IL12B 3’UTR results in lower IL-12 production, IFN$\gamma$ levels will be affected and a cytokine imbalance may lead to immune activation. Proportions of activating and inhibitory killer-cell immunoglobulin-like receptor (KIR) genes were not associated with CMV disease in African American HIV patients, but investigations of KIR genes in combination with KIR-ligands may clarify these results as NK cell function is dependent on KIR:ligand interactions. Increasing the sample size cohort and investigating haplotype as well as functional studies into these SNPs and genes may increase our understanding of susceptibility to CMV disease.

One limitation of the genetic susceptibility studies within this thesis was the need for a larger samples of patients and controls with detailed phenotyping data. Sample size calculations were not performed prior as the cohorts were drawn from pre-existing collaborative studies. We maintain their validity as pilot studies investigating the possibility of trends worthy of further study rather than definitive effect/association of the SNP or linked gene with susceptibility to the chronic diseases.

To summarise, TNFA-1031 may exert various effects on the immune system of HIV patients co-infected with TB, predisposing to low nadir CD4 T cell counts in CMV seropositive HIV patients, as well as NTM disease. Carriage of alleles of TNFA polymorphisms incorporating TNFA-1031 may have altered expression of TNF levels. Likewise, other alleles of cytokine polymorphisms such as IL10-1082 may affect its respective IL-10 production. Hence this fits the model where an imbalance may result from these altered expression of TNF and IL-10 within the immune system leading to chronic inflammation which has been closely associated with long-term morbidity and mortality in patients, potentiating persistent immune activation and leading to clinical exacerbations.
2 CMV Ab titres and CD8 T cell responses to CMV remain elevated and associate with greater immunosenescence in older HIV patients who began ART with very low CD4 T cell counts and achieved viral suppression

I investigated the immunological “footprint” of CMV on the host immune system via its effects on T-cell senescence. Investigations were undertaken in a unique cohort of CMV seropositive HIV patients who began ART with advanced immune deficiency over 12 years earlier and maintained undetectable plasma HIV ribonucleic acid (RNA) levels for more than a year.

Here, I demonstrate that humoral responses to CMV are still elevated even after more than 12 years on ART. Levels of CMV antibody titres [lysate, glycoprotein B (gB) and immediate-early 1 (IE1)] remain high in CMV seropositive HIV patients compared to healthy CMV seropositive controls. This was also apparent when CMV seropositive and seronegative controls were compared. In HIV patients, CD8 T cell IFNγ responses against VLE peptide of the CMV IE1 protein persisted. This is in agreement with Stone et al observing elevated CD8 T cells IFN\(\gamma\) responses to VLE in a cohort of HIV patients stable on ART when compared to controls 76. CD4 T cell IFN\(\gamma\) responses to the CMV pp65 peptide pool were elevated in HIV patients when compared to CMV seropositive healthy controls. Naeger et al demonstrated that CMV-specific T cells (IE and pp65) remain elevated during long-term ART in HIV seropositive patients 77 compared to HIV seronegative individuals. In a study of very old subjects (>85 years old) not infected with HIV, Vescovini et al demonstrated a marked increase in IFN\(\gamma\) producing CD4 and CD8 T cells responding to CMV pp65 and CMV IE1 VLE peptide 78. Here, raised levels of sBAFF in CMV seropositive HIV patients may be a presentation of the effects of HIV itself on the patients’ immune system, however it may also contribute to the elevated levels of CMV antibodies in older HIV patients.
In longitudinal studies of the elderly whose immune system is not impaired by HIV, CMV seropositivity is a strong indicator within the “immune risk profile” and is associated with mortality. CMV seropositivity has been linked to “accelerated ageing.” Here, age also correlated with enhanced CD57+CD45RA+CD27- expression on CD8 T-cells. This senescent phenotype also exhibited associations with antibodies against the CMV IE1 protein and CD4 T cell IFNγ response to CMV lysate. CMV seronegative healthy controls did not exhibit elevated expression of immunosenescent markers on CD4 and CD8 T cells. This demonstrates that CMV seropositivity does play a part in the immune ageing process and further worsens the impairment caused by HIV. HIV and ageing share striking similarities such as frailty, neurocognitive decline and chronic viral reactivation.

In summary, increased CD8 T cell IFN γ responses to CMV pp65 and CMV IE1 VLE peptide, as well as antibodies reactive with CMV lysate, CMV gB and CMV IE1, persist at high levels even after more than 12 years of effective combination antiretroviral therapy. This heightened overall CMV-specific inflammatory response in antiretroviral treated HIV patients suggests possible sub-clinical CMV replication. Another likely scenario could be an increase in immunological response to an otherwise basal level of CMV replication as a consequence of the loss of immunoregulatory cells due to HIV infection. CMV is an important pathogen responsible for potentiating a robust immune activation response leading to immunosenescence, as shown by increased numbers of the senescent phenotype, CD57+CD45RACD27- expression on CD8 T cells in this cohort.
3 CONCLUSIONS FROM THIS STUDY

SNPs and haplotypes affecting cytokine or chemokine production affect several disease states characterised by chronic inflammation but the effects between the diseases studied are varied depending on the ethnicity. This shows that genotypes critical in one population differed from those affecting susceptibility to NTM, an IRD event or developing CMV end-organ disease in another population. The findings of this thesis, collectively, indicate that the TNFA-1031, IL10-1082 and SLC11A1 D543N SNPs may exert influence on susceptibility to TB-IRD, NTM and CMV diseases.

Overall, TNFA-1031 stood out as an important SNP associated with susceptibility to TB-IRD, NTM and CMV disease. Haplotype studies into the TNF block haplotypes which includes TNFA-308 and other SNP within the promoter region should be studied in larger cohorts of HIV infected patients co-infected with TB or CMV. Previous investigations demonstrated TNF block haplotypes incorporating TNFA-1031 associated with Chinese, Malays and Caucasian HIV sensory neuropathy (SN) patients but this was not observed in African HIV patients, where SN was characterised by different haplotypes. IL10-1082 is often investigated as a haplotype in conjunction with IL10-592 and IL10-819. Its associations with IL-10 levels in mycobacterial disease and CMV patients warrants further investigations. SLC11A1 D543N association with CMV and IL28B-rs8099917 with NTM have never been described before. Functional studies of the outcomes and effects of these SNP and respective haplotypes will further explicate the pathogenesis of the disease and inflammatory etiology and aid in development of novel therapeutic strategies.
The cohort recruited for my study in Chapter 6 was unique, whereby the HIV patients were older and began ART 12 years earlier during advanced immunodeficiency. Investigating immunological markers of CMV as well as immunosenescence in CMV seropositive HIV negative individuals, allowed for observation in a non-immune-impaired cohort. I established that CMV leaves a long-lasting effect on the immune system of HIV patients even after many years on ART. Extending this study in a cohort to include CMV-seronegative HIV patients, CMV-DNA viral assessments and evaluation of the phenotype and function of CMV-specific CD8 T cells can further elucidate the mechanisms behind CMV reactivation promoting accelerated ageing of the immune system.

Host genetic polymorphisms may influence susceptibility to mycobacterial disease and/or viral reactivation, which exacerbates inflammation, in turn, driving persistent/chronic immune activation. The immune system needs to maintain a delicate balance of cytokines to mount a successful defence against pathogens. Host genetic polymorphisms may alter how the body resolves inflammation, leading to persistent immune activation and therefore potentiating "accelerated" ageing.

In conclusion, associations and correlations between these genetic and immunological markers with clinical presentations potentiate the identification of biomarkers, and susceptibility to chronic immune diseases. Furthermore, these markers may aid better understanding of the pathways involved in the pathogenesis of these diseases, leading to development of better diagnostic algorithms for clinicians.
4 REFERENCES


