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EXPLORING THE INTERPLAY OF CHEMOKINE RECEPTORS CCR5 AND CXCR6 IN MECHANISMS OF NATURAL CONTROL IN HIV-1-INFECTED BLACK SOUTH AFRICANS

Gemma Whitney Koor

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Virology.

Johannesburg, 2023.

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Declaration

I, Gemma Whitney Koor (Student number: 968332) am a student registered for the degree of PhD (Virology) in the academic year 2023.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
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- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

Signature:  Date: 14 June 2023

This work is dedicated to my Gags, whose unwavering support, curiosity and zest for life will inspire me always.

Publications arising from this study

Koor GW, Paximadis M, Picton ACP, Karatas F, Loubser SA, He W, Ahuja SK, Chaisson RE, Martinson N, Ebrahim O, Tiemessen CT. *Cis*-regulatory genetic variants in the CCR5 gene and natural HIV-1 control in black South Africans. Clin Immunol. 2019 Aug;205:16-24. doi: 10.1016/j.clim.2019.05.009. Epub 2019 May 15. PMID: 31100442; PMCID: PMC6646080. (Chapter 3)

Role in publication: acquisition of data, interpretation of data and drafting of article.

Permissions from co-authors to include published work in this thesis have been granted.

Presentations arising from this study

Koor GW, Paximadis M, Picton ACP, Karatas F, Loubser SA, He W, Ahuja SK, Chaisson RE, Martinson N, Ebrahim O, Tiemessen CT**.** CCR5 Expression-Related Gene Variants and Natural HIV-1 Control in Black South Africans. Oral presentation at: The University of Witwatersrand Health Sciences Research Day; 2018 Sep 6; Johannesburg, South Africa. (Chapter 3)

Koor GW, Paximadis M, Picton ACP, Karatas F, Loubser SA, He W, Ahuja SK, Chaisson RE, Martinson N, Ebrahim O, Tiemessen CT**.** CCR5 Expression-Related Gene Variants and Natural HIV-1 Control in Black South Africans. Poster presented at: HIV Research for Prevention (HIVR4P); 2018 Oct 21-25; Madrid, Spain. (Chapter 3)

Abstract

In sub-Saharan Africa, HIV-1 is a significant cause of morbidity and mortality. However, research remains primarily focused on North American and European population groups, who have remarkably different genetic backgrounds to individuals from sub-Saharan Africa. HIV-1 controllers represent a model of HIV-1 functional cure, with some individuals able to control viral replication, and some able to sustain immune function in the presence of high viral loads, both in the absence of antiretroviral therapy (ART). The chemokine receptors CCR5 and CXCR4 are the major coreceptors HIV-1 utilises to enter cells. The use of alternative coreceptors, such as the CXCR6 coreceptor, is thought to contribute to the lower pathogenicity exhibited by the HIV-2 and SIVsmm strains. Building on previous work conducted in our research unit on these two coreceptors in South African populations, this thesis firstly describes *CCR5* genetic variants that associate with HIV-1 control or risk of progressive infection in black South Africans, and then explores constitutive expression levels of CCR5 and CXCR6 on various peripheral blood immune cell subsets in the absence of HIV-1 infection in ethnically divergent population groups. The effect of sex, age, and select *CCR5* and *CXCR6* single nucleotide polymorphisms (SNPs) on expression levels of these two receptors was also investigated.

The *CCR5* 5'UTR and 3'UTR regions were PCR-amplified and sequenced from genomic DNA extracted from 145 ART-naive black South African individuals living with HIV-1 (71 HIV-1 controllers – 23 elite controllers, 37 viraemic controllers, 11 high viral load long-term non-progressors and 74 progressors). Findings confirmed results from other studies in showing that the *CCR5* HHE haplotype is deleterious for HIV-1 disease progression, and the HHA haplotype and HHA/HHC genotype associated with protection from HIV-1 disease progression. Novel haplotypes were characterised, both in the 3'UTR and spanning the *CCR5* 5'UTR and 3'UTR. Overall, findings suggest that two *CCR5* promoter SNPs (-2459 G>A and -2135 T>C) and one *CCR5* 3'UTR SNP (+2919 T>G) may be key functional variants with regards to HIV-1 control in black South Africans.

To gain further insight into the constitutive expression of CCR5 and CXCR6 on peripheral blood immune cells and explore the relationship between select genetic variants and expression, immunophenotyping by flow cytometry was conducted using whole blood from age- and sex-matched ethnically distinct South African HIV-uninfected individuals (17 black, 21 white).

Expression levels of CCR5 and CXCR6 were assessed on CD4+ and CD8+ T cells, B cells, monocytes and NK cells, and their respective subsets. The effects of age and sex on expression levels of these two receptors was also investigated. Population-specific differences with regards to CCR5 expression on all cell types, except for B cells, were evident. Generally, black South Africans exhibited a lower expression level of CCR5 compared to white South Africans. CXCR6 expression only differed with regards to percentage of CXCR6-expressing cells, not CXCR6 density (numbers of cell surface receptors). Black individuals had a lower percentage of CXCR6-expressing CD8+ T cell subsets (naïve and effector memory) and a higher percentage of CXCR6-expressing $CD14^{+}CD16^{+}$ monocytes compared to white individuals. Overall, we found significant population-specific differences in expression levels of both CCR5 and CXCR6, multiple associations with cell activation (as measured by HLA-DR expression) and CCR5 and CXCR6 expression, and CCR5 and CXCR6 expression was positively significantly correlated on multiple cell subsets.

Furthermore, both sex and age influenced CCR5 and CXCR6 expression, however results varied widely across the two population groups studied. Sex differences were only evident in white individuals; predominantly CXCR6 expression was increased in males compared to females. Age associations with CCR5 and CXCR6 expression were also primarily found in white individuals.

Four *CCR5*-related SNPs that are associated with HIV-1 control in this or other studies (rs553615728 -4223 C>T SNP, rs1799987 −2459 G>A SNP, rs746492 +2919 T>G SNP and rs1015164 G>A SNP) were assessed for their potential association with CCR5 expression levels. The +2919 TG genotype significantly associated with a higher percentage of CCR5-expressing total CD8+ T cells, transitional memory and terminally differentiated CD8+ T cells compared to the GG genotype. The +2919 GG genotype associated with a lower percentage of CCR5-expressing B cells compared to the TT and TG+TT genotypes, however, only in white South Africans. The +2919 TG and TG+TT genotypes associated with significantly higher CCR5 density on all CD8+ T cell subsets, except for naïve CD8+ T cells, when compared to the GG genotype.

When evaluating two *CXCR6* genetic variants previously associated with HIV-1 viraemic control (rs2234355 G>A and rs2234358 G>T) in relation to CXCR6 expression, possession of the rs2234355 SNP GA genotype associated with lower CXCR6 expression on select CD4+ and CD8+ T cell subsets as well as on B cells, while possession of the rs2234358 SNP TT genotype associated with higher CXCR6 expression on multiple cell types, primarily in white South Africans.

Possession of the -358TT/+355GA genotype combination associated with lower CXCR6 expression on select subsets of CD4+ T cells and monocytes.

In summary, this study provides information on genetic variation in the *CCR5* gene in a South African context, describes genetic variants associating with HIV-1 control in black South Africans, adds novel insight into constitutive CCR5 and CXCR6 expression levels on CD4+ and CD8+ T cells, B cells, monocytes and NK cells in HIV-1-uninfected black and white South Africans, and describes the potential associations of select genetic variants and expression. Black and white individuals differed in their baseline expression levels of CCR5 or CXCR6, which was partly driven by host genetic factors that were explored. This work highlights the importance of considering effects of ethnicity, age, and sex in any studies addressing any immune molecules in relation to differential HIV-1 outcomes of infection susceptibility/protection, disease progression, or HIV-1 virological control on antiretroviral therapy. Although conducted on small numbers of individuals, these variables clearly influenced constitutive expression of CCR5 and CXCR6, and further population-specific studies are warranted to gain further insights. Findings from this study have implications for risk of acquisition of HIV-1 infection and for disease progression in people living with HIV-1. Understanding the role of these molecules is important for informing strategies for both HIV-1 prevention and HIV cure.

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Table of Contents

Declaration i

Dedication ii

Publications arising from this study iii

Presentations arising from this study iii

Abstract iv

Acknowledgements vii

Table of Contents viii

List of Abbreviations xvii

List of Figures xxi

List of Tables xxvi

1. Introduction 1

1.1 Classification and distribution of HIV 1

1.2 Prevalence and disproportionate burden of HIV-1 2

1.3 HIV-1 virology 3

1.3.1 HIV-1 gene structure and function 3

1.3.2 HIV-1 cell entry and life cycle 4

1.3.3 HIV-1 interaction with the immune system 6

1.4 Chemokine receptors and chemokines 7

1.4.1 HIV-1 and chemokine receptor usage 7

1.4.2 Chemokines and ligands 8

1.5 Antiretroviral therapy 10

1.6 Natural control of HIV-1 11

1.7 Models of HIV-1 cure and mechanisms of control 13

1.7.1 HIV-1 virological fitness 13

1.7.2 Host factors 14

1.8 Chemokines and HIV-1 control 14

1.8.1 CCR2 14

1.8.2 CCR5 15

1.8.2.1 CCR5 ligands 15

1.8.2.2 CCR5 expression 15

1.8.2.3 *CCR5* genetic polymorphisms 16

1.8.3 CXCR6 18

1.8.3.1 *CXCR6* genetic polymorphisms 19

1.9 Regulation of gene expression 20

1.10 HIV-2 and SIVsmm as models of durable control 21

1.10.1 HIV-2 21

1.10.2 SIV 23

1.10.2.1 Natural versus non-natural hosts 23

1.10.2.2 SIV preferential coreceptor-usage 25

1.11 Study rationale 26

1.12 Aim of study 27

1.13 Specific objectives 27

2. Materials and Methods **28**

2.1 Sample population 28

2.1.1 People living with HIV-1 28

2.1.2 Healthy controls 29

2.1.3 Ethics 29

2.2 Standard polymerase chain reaction (PCR) amplification of *CCR5* 5'UTR and 3'UTR 29

2.3 Sequencing of *CCR5* 5'UTR and 3'UTR 30

2.4 Sequence analysis 31

2.5 *CCR5* haplotype assignment 31

2.6 Single nucleotide polymorphism (SNP) genotyping 32

2.7 Flow cytometry 37

2.7.1 Panel design 37

2.7.2 Whole blood staining 38

2.7.3 Quantitation 39

2.7.4 Flow cytometry quality control and standardization 39

2.7.4.1 CS&T Beads 39

2.7.4.2 Rainbow beads 39

2.7.4.3 Compensation controls 41

2.7.5 Gating controls 43

2.7.5.1 Fluorescence Minus One (FMO) controls 43

2.7.6 Gating strategies 43

2.7.6.1 CD4+ T cells 44

2.7.6.2 CD8+ T cells 46

2.7.6.3 B cells 47

2.7.6.4 Monocytes 48

2.7.6.5 NK cells 49

2.8 Assessing the effect of *CCR5* and *CXCR6* SNPs on expression levels 50

2.9 Flow cytometry data analysis 50

2.10 Statistical analyses 50

3. *Cis*-regulatory genetic variants in the *CCR5* gene and natural HIV-1 control in black South Africans 51

3.1 Introduction 51

3.2 Materials and Methods 52

3.2.1 Sample population 52

3.2.2 Standard polymerase chain reaction (PCR) amplification and sequencing of *CCR5* 5'UTR and 3'UTR 52

3.2.3 Sequence analysis 52

3.2.4 *CCR5* haplotype assignment 52

3.2.5 Single nucleotide polymorphism genotyping 52

3.2.6 Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium 53

3.2.7 Analysis and statistics 53

3.3 Results 53

3.3.1 *CCR5* 5'UTR: variability and HIV-1 control 53

3.3.2 *CCR5* 3'UTR: variability and HIV-1 control 59

3.3.3 Haplotypes spanning the *CCR5* 5'UTR and 3'UTR 62

3.3.4 Potential markers for HIV-1 disease progression 64

3.4 Discussion 65

4. CCR5 and CXCR6 expression in two ethnically distinct South African population groups 70

4.1 Introduction 70

4.2 Materials and Methods 72

4.2.1 Sample population 72

4.2.2 Flow cytometry 72

4.2.3 Statistical analyses 72

4.3 Results 72

4.3.1 CCR5 and CXCR6 expression differences 72

4.3.2 T cell memory subset proportions differ between black and white South Africans 74

4.3.3 CCR5 and CXCR6 expression on cell subsets 75

4.3.3.1 CD4+ T cells 75

4.3.3.2 CD8+ T cells 77

4.3.3.3 Monocytes 78

4.3.3.4 NK cells 79

4.3.4 Ratios and correlations: CCR5 and CXCR6 expression 82

4.3.4.1 Ratio of CCR5 to CXCR6 expression 82

4.3.4.2 Correlation between CCR5 and CXCR6 expression 85

4.3.5 Activated cells, ethnicity and CCR5 and CXCR6 expression 87

4.3.5.1 Expression differences on HLA-DR+ versus HLA-DR- cells 88

4.3.5.2 Expression differences between population groups 89

4.3.5.3 Activated CD4+ and CD8+ T cells correlated with CCR5 and CXCR6 expression 92

4.3.6 Summary of findings 96

4.4 Discussion 98

5. Influence of sex and age on CCR5 and CXCR6 expression in two ethnically distinct South African population groups **107**

5.1 Introduction 107

5.2 Materials and Methods 108

5.2.1 Sample population 108

5.2.2 Flow Cytometry 108

5.2.3 Statistical analyses 108

5.3 Results 108

5.3.1 Sex differences 108

5.3.1.1 CCR5 expression and sex 109

5.3.1.2 CXCR6 expression and sex 109

5.3.2 Age effects 115

5.3.2.1 CCR5 expression and age 115

5.3.2.2 CXCR6 expression and age 115

5.3.2.3 Comparing CCR5 and CXCR6 findings 116

5.4 Discussion 118

6. Select *CCR5* SNPs have varying associations with CCR5 expression levels in two ethnically distinct South African population groups **122**

6.1 Introduction 122

6.2 Materials and Methods 123

6.2.1 Sample population 123

6.2.2 Single nucleotide polymorphism (SNP) genotyping 124

6.2.3 Flow cytometry 124

6.2.4 Statistical analyses 124

6.3 Results 124

6.3.1 Allelic and genotypic representation of *CCR5* SNPs 124

6.3.2 *CCR5* -4223 C>T SNP and CCR5 expression 125

6.3.3 *CCR5* -2459 G>A SNP and CCR5 expression 125

6.3.4 *CCR5* +2919 T>G SNP and CCR5 expression 127

6.3.4.1 Frequency of CCR5 expression and the +2919 SNP 127

6.3.4.2 CCR5 density and the +2919 SNP 133

6.3.5 *CCR5* rs1015164 G>A SNP and CCR5 expression 139

6.4 Discussion 139

7. The *CXCR6* rs2234355 and rs2234358 SNPs associate with altered CXCR6 expression levels in two ethnically distinct South African population groups in the absence of HIV-1 142

7.1 Introduction 142

7.2 Materials and Methods 143

7.2.1 Sample population 143

7.2.2 Single nucleotide polymorphism (SNP) genotyping 144

7.2.3 Flow cytometry 144

7.2.4 Statistical analyses 144

7.3 Results 144

7.3.1 *CXCR6* rs2234355 G>A SNP and CXCR6 expression 144

7.3.2 *CXCR6* rs2234358 G>T SNP and CXCR6 expression 147

7.3.3 *CXCR6* -358TT/+355GA and CXCR6 expression 150

7.4 Discussion 150

8. Summarising discussion and conclusions 154

8.1 *CCR5* polymorphisms associating with HIV-1 natural control in black South Africans 155

8.2 Differences in proportions of T cells in black and white South Africans 155

8.3 CCR5 and CXCR6 constitutive expression differences between black and white South Africans 156

8.3.1 CCR5 expression differences 156

8.3.1.1 $CD14^{+}CD16^{bright}$ monocytes 157

8.3.1.2 $CD56^{dim}CD16^{-}$ NK cells 158

8.3.1.3 CD8+ T cells 158

8.3.1.4 Central memory CD4+ T cells 158

8.3.1.5 CCR5-CXCR4 co-receptor switching and HIV-1 pathogenesis 159

8.3.1.6 CCR5-based cure strategies 160

8.3.2 CXCR6 expression differences 160

8.3.2.1 Naïve and effector memory CD8+ T cells 160

8.3.2.2 $CD14^{+}CD16^{+}$ monocytes 161

8.4 Relationship between CCR5 and CXCR6 162

8.4.1 CCR5 and CXCR6 expression ratios 162

8.4.2 CCR5 and CXCR6 expression correlations 163

8.5 Activation and effect on CCR5 and CXCR6 expression 163

8.6 Impact of sex and age on CCR5 and CXCR6 expression 164

8.7 *CCR5* and *CXCR6* gene variants and expression 165

8.7.1 *CCR5* variants 165

8.7.2 *CXCR6* variants 168

8.8 Study limitations 168

8.9 Conclusion and future recommendations 169

REFERENCES **171**

APPENDIX A: Ethical Clearance **202**

APPENDIX B: Turnitin originality report **203**

°C	Degrees Celsius
7TMR	Seven-transmembrane G protein-coupled receptors
AIDS	Acquired immunodeficiency syndrome
AGM	African green monkey
APC	Allophycocyanin
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
bp	Base pair
BV	Brilliant Violet
cART	Combination anti-retroviral therapy
CCR	C-C chemokine receptor
CCR5Δ32	CCR5 delta 32 (32 bp deletion)
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CI	Confidence interval
CM	Central memory
CMV	Cytomegalovirus
CNS	Central nervous system
Cpg	Cytidine phosphate guanidine
Cq	Cycle threshold
CRF	Circulating recombinant form
CXCR4	CXC-chemokine receptor 4
CXCR6	CXC-chemokine receptor 6
DNA	Deoxyribonucleic acid
EC	Elite controller
EDTA	Ethylenediaminetetraacetic acid
EM	Effector memory
Env	HIV viral envelope glycoprotein
eQTL	Expression quantitative trait loci

FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
g	g-force
GWAS	Genome-wide association study
HAART	Highly active anti-retroviral therapy
HC	Healthy control
HCV	Hepatitis C virus
HIC	HIV-1 controller
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA	Human leukocyte antigen
HVL LTNP	High viral load long-term non-progressor
IFN-γ	Interferon gamma
IL-2	Interleukin 2
Indel	Small insertion or deletion of bases
ITAC	Interferon–inducible T cell alpha chemoattractant
IQR	Interquartile range
kb	Kilobases
LD	Linkage disequilibrium
LNA	Locked nucleic acid
lncRNA	Long non-coding RNA
LTNP	Long-term non-progressor
LTR	Long terminal repeat
MDM	Monocyte-derived macrophage
MFI	Mean/median fluorescence intensity
MGB	Minor groove binder
$MgCl_2$	Magnesium chloride
MIF	Macrophage migration inhibitory factor
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
Mt	Mutant

NAFLD	Non-alcoholic fatty liver disease
NFQ	Non-fluorescent quenchers
ng	Nanogram
NK	Natural killer
NKT	Natural killer T
nM	Nanomolar
NTC	No template control
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll-A protein
PIC	Pre-integration complex
ρmol	Picomole
qPCR	Quantitative real-time polymerase chain reaction
rCV	Robust coefficient of variation
RNA	Ribonucleic acid
RPA	Relative promoter activity
rpm	Revolutions per minute
SIV	Simian immunodeficiency virus
SIVcpz	Simian immunodeficiency virus in wild chimpanzees
SIVsmm	Simian immunodeficiency virus in sooty mangabeys
SIVmac	Simian immunodeficiency virus in macaques
SM	Sooty mangabey
SNP	Single nucleotide polymorphism
SSC	Side scatter
Tc1	Type 1 cytotoxic
TD	Terminally differentiated
Th1	Type 1 helper
TM	Transitional memory
TNF	Tumour-necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand

µl	Microliter
UTR	Untranslated region
V3	Third variable region
VC	Viraemic controller
VL	Viral load
VLS	Viral load setpoint
WT	Wild-type

List of Figures

Figure 1.1	The origin of HIV-1 and HIV-2.	1
Figure 1.2	HIV-1 genes and summary of functions.	3
Figure 1.3	Genomic organization of HIV-1 and HIV-2 and related primate lentiviruses.	4
Figure 1.4	Illustration of the HIV-1 replication cycle.	6
Figure 1.5	Chemokine receptors and their ligands.	9
Figure 1.6	HIV-1 antiretroviral drugs and target stages of HIV-1 life cycle.	10
Figure 1.7	The categories of individuals with differing rates of HIV-1 disease progression, based on viral load (RNA copies/ml) and/or CD4+ T cell count (cells/µl).	12
Figure 1.8	Polymorphisms defining the nine CCR5 haplotypes.	17
Figure 2.1	Polymorphisms forming each CCR5 promoter haplotype.	32
Figure 2.2	Representative C_q shift amplification plots for a WT/WT, WT/Mt and Mt/Mt genotype.	35
Figure 2.3	Histograms depicting a signal detection from rainbow beads in all channels used in the CCR5 and CXCR6 panels.	41
Figure 2.4	Representative histograms of unstained (green) and stained (red) compensation beads for all fluorochromes used in the CCR5 and CXCR6 panels.	42
Figure 2.5	Representative gating strategy for naïve and memory CD4+ T cell subsets.	44
Figure 2.6	Representative gating strategy for naïve and memory CD8+ T cell subsets.	46
Figure 2.7	Representative gating strategy for B cells.	47
Figure 2.8	Representative gating strategy for monocyte subsets.	48
Figure 2.9	Representative gating strategy for natural killer cell subsets.	49

Figure 3.1	Bar graph showing the allelic frequencies (%) of previously defined CCR5 promoter haplotypes in black South Africans living with HIV-1.	54
Figure 3.2	Bar graph showing the genotypic frequency of 5'UTR-2SNP-hap (-2459 G>A and -2135 T>C) in black South African controllers, controller subgroups (ECs, VCs and HVL LTNPs) and progressors.	56
Figure 3.3	Linkage disequilibrium (LD) plot depicting the linkage between CCR5 3'UTR variants.	61
Figure 3.4	a) CCR5 polymorphisms that form each haplotype and b) bar graph showing the allelic frequencies (%) of CCR5 3'UTR haplotypes in black South African controllers, controller subgroups (ECs, VCs and HVL LTNPs) and progressors.	62
Figure 3.5	Polymorphisms that form haplotypes spanning the CCR5 5'UTR and 3'UTR in black South African controllers, controller subgroups (ECs, VCs and HVL LTNPs) and progressors.	63
Figure 3.6	Summary of the p values of all significant CCR5 genetic markers predictive of HIV-1 progression in black South Africans (HIV-1 controllers vs progressors).	65
Figure 4.1	Graphs showing CCR5 (left) and CXCR6 (right) expression on lymphocyte subsets in black and white individuals.	74
Figure 4.2	Graphs showing T cell subset proportions of (a) total CD4+ T cells and (b) total CD8+ T cells in black and white South Africans.	75
Figure 4.3	Graphs showing CCR5 (left) and CXCR6 (right) expression on CD4+ T lymphocyte subsets in black and white South African individuals.	76
Figure 4.4	Graphs showing CCR5 (left) and CXCR6 (right) expression on CD8+ T lymphocyte subsets in black and white South African individuals.	77

Figure 4.5	Graphs showing CCR5 (left) and CXCR6 (right) expression on monocyte subsets in black and white South African individuals.	79
Figure 4.6	Graphs showing CCR5 (left) and CXCR6 (right) expression on NK cell subsets in black and white South African individuals.	80
Figure 4.7	Graphs showing the ratio of frequency (left) and density (right) of CCR5 to CXCR6 expression for the gross subset of (a) CD4+ T cells, (b) CD8+ T cells, (c) B cells, (d) monocytes and (e) NK cells.	82
Figure 4.8	Graphs showing the ratio of frequency (left) and density (right) of CCR5 to CXCR6 expression for the (a) CD4+ T cell subsets, (b) CD8+ T cell subsets, (c) monocyte subsets and (d) NK cell subsets.	84
Figure 4.9	Graphs showing the percentage of HLA-DR+ (a) CD4+ T cells, (b) CD8+ T cells, (c) B cells, (d) monocytes and (e) NK cells in black and white South Africans.	88
Figure 4.10	CCR5 and CXCR6 expression on HLA-DR^{+} and HLA-DR^{-} lymphocyte subsets in black and white South African individuals.	91
Figure 4.11	Heatmap showing correlations between % HLA-DR+ CD4+ T cells and CCR5 and CXCR6 expression on CD4+ T cells and subsets CD8+ T cells and subsets, CD19+ B cells, CD14+ monocytes and subsets and CD56+ NK cells and cell subsets.	93
Figure 4.12	Heatmap showing correlations between % HLA-DR+ CD8+ T cells and CCR5 and CXCR6 expression on CD4+ T cells and subsets CD8+ T cells and subsets, CD19+ B cells, CD14+ monocytes and subsets and CD56+ NK cells and cell subsets.	95
Figure 4.13	Summary of significant ethnic differences with regards to CCR5 and CXCR6 expression, ratios of CCR5 to CXCR6 expression and correlations of CCR5	97

and CXCR6 expression on CD4+ T cell subsets, CD8+ T cell subsets, B cells, monocyte subsets and NK cell subsets.

Figure 5.1 Summary of significant sex effects on CCR5 and CXCR6 expression on total CD4+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD4+ T cell subsets, total CD8+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets, CD19+ B cells, total CD14+ monocytes and monocyte subsets and total CD56+ NK cell subsets. 110

Figure 5.2 Heatmap showing correlations between age and CCR5 and CXCR6 expression on (a) total CD4+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD4+ T cell subsets (b) total CD8+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets, (c) CD19+ B cells, (d) total CD14+ monocytes and monocyte subsets and (e) total CD56+ NK cell subsets. 117

Figure 6.1 Summary of significant changes to percentage of CCR5-expressing CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells associated with -2459 SNP genotype. 126

Figure 6.2 Summary of significant changes to percentage of CCR5-expressing CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells associated with +2919 SNP genotype. 130

Figure 6.3	Summary of significant changes to percentage of CCR5-expressing cells associated with +2919 SNP genotype surviving false discovery rate (FDR) and Bonferroni multiple comparison corrections.	132
Figure 6.4	Summary of significant changes to CCR5 density on CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells associated with +2919 SNP genotype.	134
Figure 6.5	Summary of significant changes to CCR5 density associated with +2919 SNP genotype surviving false discovery rate (FDR) and Bonferroni multiple comparison corrections.	136
Figure 6.6	Frequency (left) and density (right) of CCR5 expression according to the +2919 SNP genotypes for total, naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets in white South Africans and in the total group.	137
Figure 6.7	+2919 SNP genotype (GG vs TG+TT) and CCR5 density on total, naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets in white South Africans and in the total group.	138
Figure 7.1	Summary of CXCR6 expression changes associated with the rs2234355 genotype.	146
Figure 7.2	Summary of CXCR6 expression changes associated with the rs2234358 genotype.	149

List of Tables

Table 1.1	Comparison of immune response to infection with HIV-1 and HIV-2.	22
Table 1.2	Main features of SIV infection of natural hosts compared to non-natural hosts.	24
Table 2.1	Characteristics of study cohort of people living with HIV-1.	28
Table 2.2	Primers used for PCR and sequencing of CCR5 5'UTR and 3'UTR regions.	31
Table 2.3	C_q qPCR assay primer sequences, run settings and SYBR Green reagent used.	36
Table 2.4	Antibodies used in flow cytometry panels.	38
Table 3.1	Genotypic frequencies of CCR5 promoter haplotypes in black South African controllers, controller subgroups and progressors.	55
Table 3.2	Comparison of the genotypic and allelic representation of CCR5 5'UTR-2SNP-hap (-2459 G>A and -2135 C>T) and CCR5 3'UTR +2919 T>G SNP in controllers, controller subgroups and progressors.	57
Table 3.3	Genotypic frequencies of SNPs found in the CCR5 5'UTR and the CCR5 3'UTR in black South African controllers, controller subgroups and progressors.	58
Table 4.1	Comparison of CCR5 and CXCR6 expression (frequency and density) on $CD56^{bright}$ versus $CD56^{dim}$ and CD16+ versus CD16- subsets of NK cells for the total group.	81
Table 4.2	Correlation analyses results for CCR5 versus CXCR6 expression on CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells.	86
Table 5.1	Percentage of CCR5-expressing cells differences by sex.	111
Table 5.2	CCR5 density differences by sex.	112
Table 5.3	Percentage of CXCR6-expressing cells differences by sex**.**	113
Table 5.4	CXCR6 density differences by sex.	114
Table 6.1	Genotypic and allelic frequencies of the -4223 C>T, -2459 G>A, +2919 T>G and rs1015164 G>A polymorphisms in black and white South Africans.	124

CHAPTER ONE

1. Introduction

1.1 Classification and distribution of HIV

HIV-1 and HIV-2 are lentiviruses of the family Retroviridae and are the causative agents of acquired immunodeficiency syndrome (AIDS). HIV is believed to have arisen from multiple species crossover events of simian immunodeficiency viruses (SIV) from African primates. It is widely accepted that SIV from chimpanzees gave rise to HIV-1 group M (Major), the primary virus group of the original AIDS pandemic, while HIV-2 is closely related to the SIV that infects sooty mangabeys (1,2) (Figure 1.1). The three other HIV-1 groups are N (New/Non-M/Non-O), O (Outlier) and P, the newest group of HIV-1 (3).

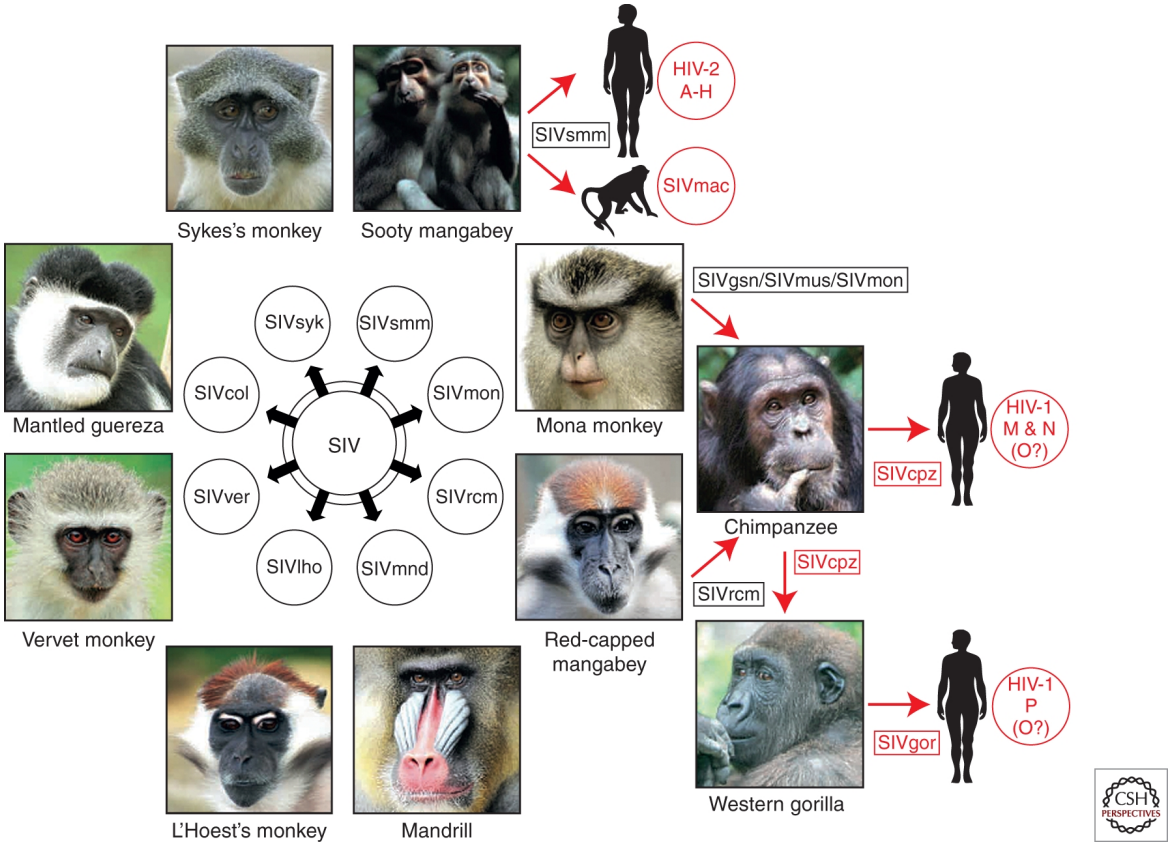


Figure 1.1 - The origin of HIV-1 and HIV-2. Simian immunodeficiency viruses (SIVs) are labelled with a suffix to denote their primate species of origin (e.g., SIVsmm from sooty mangabeys). Numerous SIVs have crossed the species barrier to great apes and humans, producing new pathogens. Known examples of cross-species transmissions and the resulting viruses are highlighted in red. Reproduced from (409).

The spread of HIV-1 group M resulted in different, distinct subtypes in different geographical regions. There are nine major subtypes (A-D, F-H, J and K) and at least 48 circulating recombinant forms (CRFs) (4). The origin of HIV-1 was documented near Kinshasa (the present-day Democratic Republic of Congo) around the 1920s, spreading along a transport network to other areas in sub-Saharan Africa, West Africa, Europe and the rest of the world (5,6). This global spread resulted in a geographically defined distribution of genetically distinct viruses (5). For instance, subtype B is responsible for the majority of HIV-1 transmissions in Europe and America, and is hypothesised to have arisen from an African strain that spread to Haiti and then to the United States and other western countries (7). Subtypes A and D are thought to have originated in central Africa and are the major subtypes of the HIV-1 epidemic in eastern Africa, while subtype C is the major subtype in southern Africa, which then spread to India and other Asian countries.

1.2 Prevalence and disproportionate burden of HIV-1

Millions of people worldwide continue to be affected by the HIV-1 pandemic. Even with the advent of antiretroviral therapy (ART), AIDS continues to be a leading cause of morbidity and mortality in sub-Saharan Africa. According to UNAIDS, 38.4 million people were living with HIV globally in 2021 (https://www.unaids.org/en/resources/fact-sheet). There is a disproportionate burden of HIV in sub-Saharan Africa, which carries more than 70% of the global burden of transmission (8). East and southern Africa account for almost half of new HIV acquisitions globally (https://www.unaids.org/en/resources/fact-sheet).

Individuals living in sub-Saharan African countries are at an increased risk of acquiring HIV-1, with women and girls being the most vulnerable. In sub-Saharan Africa, four out of every five new transmissions among individuals aged 15 to 19 years old are in girls, and young women aged 15–24 years are twice as likely to be living with HIV than men. Young women also acquire HIV 5-7 years earlier than their male counterparts (8).

Poverty and food insecurity may lead to an increased risk of HIV-1 acquisition because women are subsequently behaviourally vulnerable to HIV-1 (9). Cultural norms and the financial dependence of women on their partners might also add to the challenge of insisting on sex with the use of condoms and other prevention tools. Low economic status has been found to be associated with an earlier age of first sexual experience, lower use of condoms, having a larger number of sexual partners and a higher probability of having non-consensual sex (10).

1.3 HIV-1 virology

1.3.1 HIV-1 gene structure and function

In addition to the prototypical retroviral structural genes (*gag, pol, env*), HIV-1 also encodes regulatory (*tat, rev*) and accessory genes (*vpr*, *vpu*, *vif, nef*). A summary of HIV-1 genes and their functions is shown in Figure 1.2 and an overview of the gene structures of HIV-1 and HIV-2, SIVsmm and SIVmac is shown in Figure 1.3.

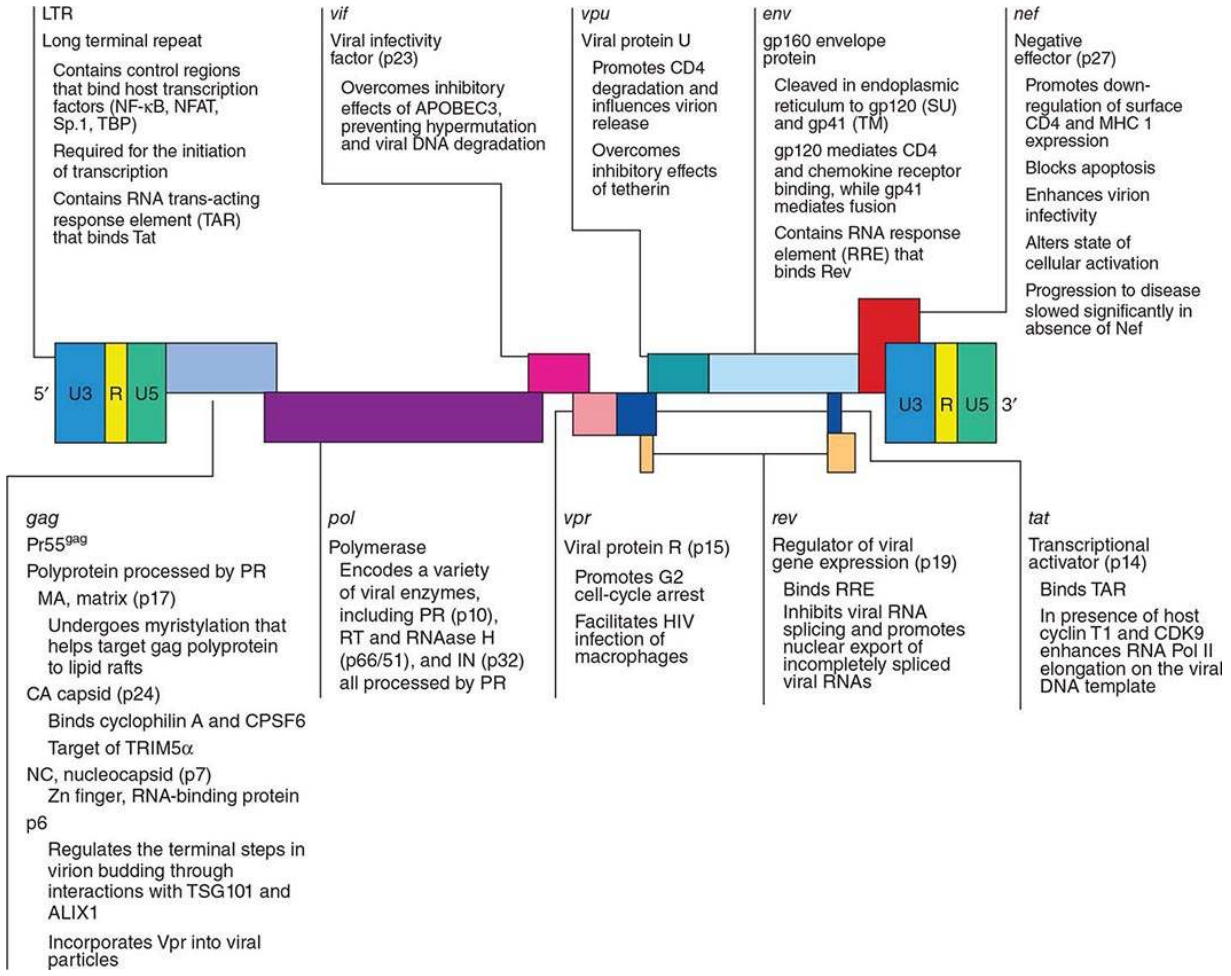


Figure 1.2 - HIV-1 genes and summary of functions. Adapted from (19).

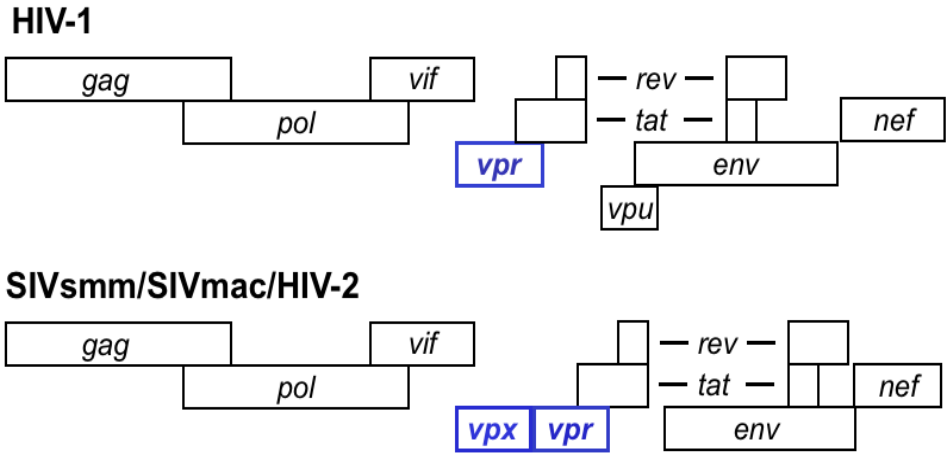


Figure 1.3 - Genomic organization of HIV-1 and HIV-2 and related primate lentiviruses. Reproduced from (410).

1.3.2 HIV-1 cell entry and life cycle

HIV is an enveloped virus. During the extracellular phase of the viral life cycle, HIV-1 genetic material and core proteins are contained within a lipid membrane, the envelope (11). The cells that HIV and SIV target are mainly determined at the level of entry. CD4+ T cells are commonly identified as the major HIV target, where HIV infects cells through the combination of the cluster of differentiation 4 (CD4) receptor and either the C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) coreceptors (12). However, HIV has also been found to enter other important immune cell types, including macrophages, dendritic cells, Langerhans cells, B cells, and granulocytes (12). In order to enter a host cell, viruses need to penetrate the plasma membrane barrier of the target cell which, for enveloped viruses, is achieved by membrane fusion (11).

Enveloped viruses contain surface glycoproteins that play an essential role in the early events of viral infection by mediating the attachment of virions to cells and by fusing of the viral and cellular membranes (11). The HIV viral envelope glycoprotein (Env) is encoded by the HIV-1 *env* gene. Env is made up of the homotrimeric protein gp160 which is cleaved into gp120 and gp41 (11,13,14). The trimeric gp120 subunit targets the CD4 T cell receptor. The CD4 receptor binding to gp120 prompts a conformational change in gp120, exposing the coreceptor (chemokine receptor) binding site. Binding to the coreceptor prompts an exposure of the hydrophobic gp41 fusion peptide

which inserts into the membrane of the host cell (11). The resulting binding of the viral and host membranes causes the fusion peptide of each gp41 to fold, forming a six-helix bundle (11,15). The formation of this bundle drives the viral and host membranes into close proximity, forming a fusion pore where the virus delivers its contents into the host cell cytoplasm (16).

HIV-1 ribonucleic acid (RNA) and proteins then enter into the cytoplasm of the target cell, where the HIV-1 RNA is transcribed into complementary DNA (cDNA), and then to double stranded DNA (dsDNA) (17). This newly synthesised DNA, in association with viral and cellular proteins, becomes what is termed the pre-integration complex (PIC). Due to the high molecular weight of the PIC, passive diffusion across the nuclear membrane is unable to occur. HIV-1 instead actively delivers its DNA into the nucleus with the help of host nuclear import machinery using a process that requires adenosine triphosphate (ATP) but is independent of cell division (17–19).

The viral DNA subsequently integrates into the host DNA and is termed the proviral DNA. The HIV-1 proviral DNA is transcribed and translated by the host cell machinery, thereby generating new virions (20). The viral polyproteins are cleaved by the protease enzyme inside the immature virion, mature Gag proteins are formed and infectious virions are produced that start the cycle again (20). A summary of the HIV-1 life cycle can be seen in Figure 1.4.

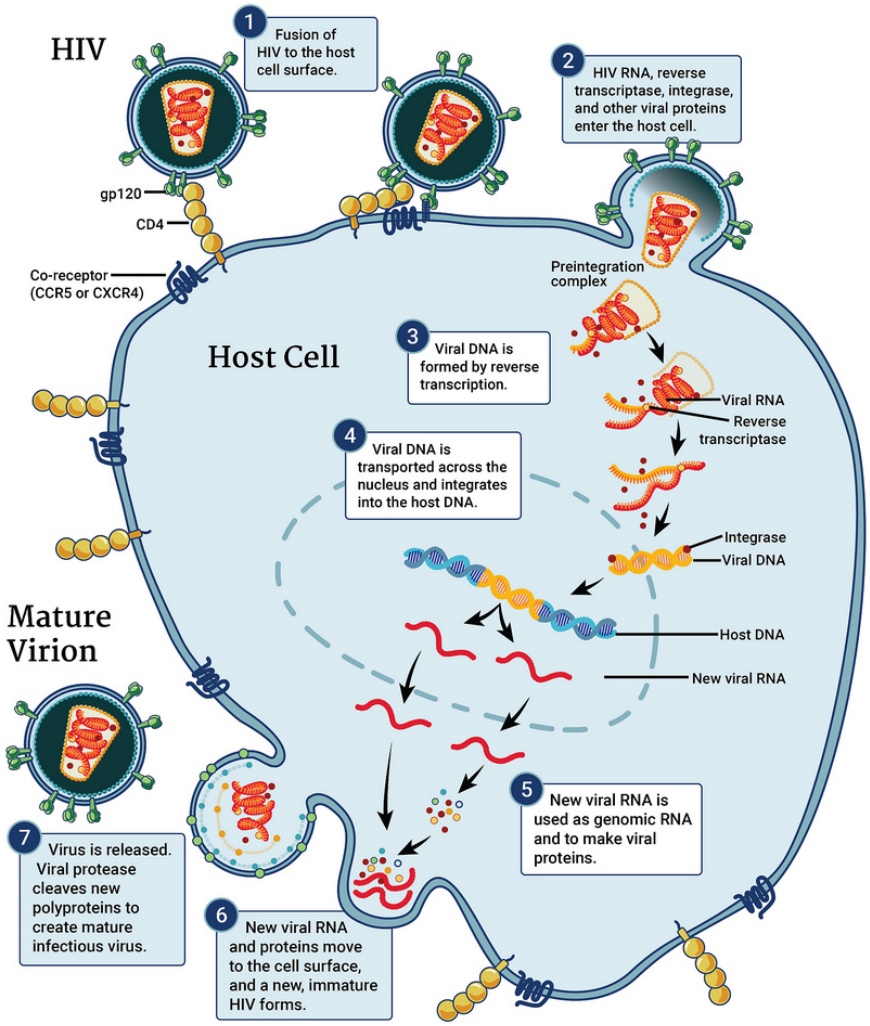


Figure 1.4 – Illustration of the HIV-1 replication cycle. The HIV-1 life cycle begins with the fusion of HIV-1 with the host cell and ends with the synthesis of mature, infectious viruses. Source: https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle.

1.3.3 HIV-1 interaction with the immune system

The immune system can be divided into two main arms, the innate immune system and the adaptive immune system. While the innate immune system is the first line of defense against pathogens and is classically responsible for non-specific protection against common bacterial/viral infections, the adaptive immune system provides protection against reinfection with the same pathogen (21,22).

The innate immune system is made up of several different cell types, including natural killer cells, mast cells, eosinophils, basophils, macrophages, neutrophils and dendritic cells, which are recruited to sites of infection through the production of cytokines and other chemical factors (23). The adaptive immune system relies on the generation of diverse antigen-specific receptors on T and B cells by somatic rearrangement in blast cells (24). When a foreign antigen is detected in the body, it is presented to the immune system in association with human leukocyte antigen (HLA) class I on infected cells and class II molecules on antigen presenting cells (25). The adaptive immune response is then initiated against the specific antigen, which includes the direct targeting and killing of infected cells by cytotoxic T lymphocytes, and the production of antibodies by stimulated B cells (24). HIV-1 has an error-prone reverse transcriptase resulting in the frequent acquisition of mutations in its genome which allows HIV-1 to evade the immune system (26,27).

1.4 Chemokine receptors and chemokines

1.4.1 HIV-1 and chemokine receptor usage

Chemokine receptors are found on the surfaces of many cells and play a central role in the development and homeostasis of the immune system by promoting cellular migration by chemotaxis, cell adhesion, and release of mediators of inflammation (28–30). Chemokine receptors are involved in all protective or destructive immune and inflammatory responses (31). They are seven-transmembrane G protein-coupled receptors (7TMRs). At the amino acid level, these 7TMRs are quite diverse and non-conserved, but general characteristics such as their seven transmembrane properties and a signaling motif in the second intracellular loop are generally conserved between receptors (32).

When chemokine receptors were identified as necessary coreceptors to allow HIV-1 entry into target cells, this greatly furthered the understanding of the pathogenesis of HIV-1. HIV-1 viruses tend to bind to CCR5 to establish a primary infection and are termed R5 viruses. As the infection evolves, in more than half of infected individuals, viruses will exhibit a change in receptor usage to CXCR4 (X4 viruses), which associates with an increased decline in CD4+ T cell count and a more rapid disease progression (31,33). CXCR4 usage tends to be limited to subtype B strains, being less common in the subtype C-driven HIV-1 transmissions in sub-Saharan Africa or South East Asia (34). While the major HIV-1 coreceptors are CCR5 and CXCR4, there are many other

coreceptors that have been shown to support HIV-1 infection and replication, including, but not limited to, CCR2b, CXCR6, GPR1 and GPR15 (35).

1.4.2 Chemokines and ligands

Chemokines are a family of cytokines, which are small proteins important in cell signaling. When injury or infection occurs, chemokines are involved in the recruitment of different leukocyte cells to these sites (36). Most chemokine receptors can be recognised by multiple ligands, while a chemokine ligand can bind to a number of different receptors (37).

Named for how the cysteines within them are arranged, there are two main chemokine ligand superfamilies (38). In the CC family, the first two cysteines near the amino terminus are located next to each other and in the CXC family there is one amino acid between them (38). The CXC chemokines are mainly involved in recruiting neutrophils while the CC chemokines recruit other leukocytes like monocytes, lymphocytes and basophils (39,40). A summary of chemokine receptors and their ligands is shown in Figure 1.5.

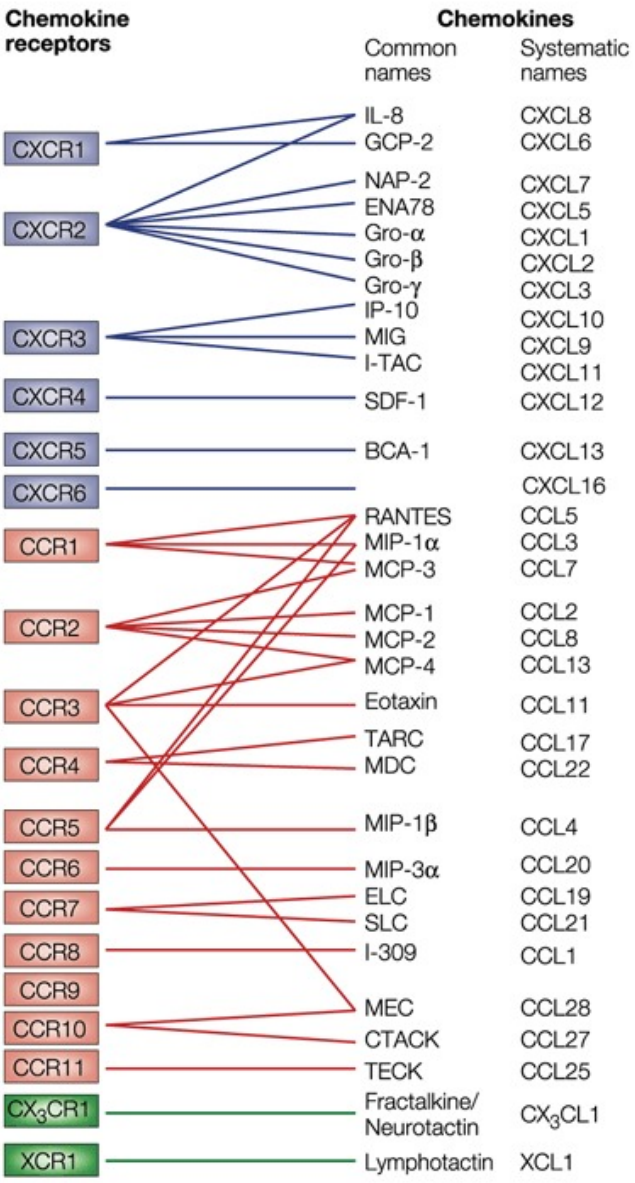


Figure 1.5 - Chemokine receptors and their ligands. The receptors for the CXC subclass are shown in blue, the receptors for the CC subclass are shown in red and the receptors for the minor subclasses (C and CX3C) are shown in green. MCP-1: monocyte chemoattractant protein 1. SDF-1: stromal derived factor 1. MEC: mucosae-associated epithelial chemokine. BCA-1: B-cell-attracting chemokine 1. CTACK: cutaneous T-cell-attracting chemokine. ELC: Epstein–Barr-virus-induced gene 1 ligand chemokine. ENA78: epithelial-cell-derived neutrophil-activating peptide 78. GCP-2: granulocyte chemotactic protein 2. Gro: growth-regulated oncogene. IL-8: interleukin 8. IP-10: interferon-inducible protein 10. I-TAC: interferon-inducible T-cell α chemoattractant. MCP: monocyte chemoattractant protein. MDC: macrophage-derived chemokine. MEC: mucosae-associated epithelial chemokine. MIG: monokine induced by interferon γ. MIP: macrophage inflammatory protein. NAP-2: neutrophil-activating peptide 2. RANTES: regulated on activation, normal T-cell expressed and secreted. SDF-1: stromal-cell-derived factor 1. SLC: secondary lymphoid-tissue chemokine. TARC: thymus and activation-regulated chemokine. TECK: thymus-expressed chemokine. Reproduced from (37).

1.5 Antiretroviral therapy

The only individuals that have attained complete remission of HIV-1 underwent haematopoietic stem-cell transplantation procedures using a donor with a homozygous mutation in the HIV coreceptor *CCR5* (*CCR5Δ32*) (41) which is not feasible for most individuals living with HIV-1. Antiretroviral therapy (ART) is currently the only treatment available for individuals living with HIV-1. Different drugs have been identified that target different stages of HIV-1 replication (Figure 1.6) (42). When HIV-1 is replicating, for every 1000 to 10 000 nucleotides synthesised, approximately one mutation is generated (43–45). With the HIV-1 genome being approximately 10 000 nucleotides in length, one to ten mutations are introduced into each viral genome with every replication cycle. These mutations contribute to immune escape and ART resistance. To lessen the likelihood of HIV-1 developing resistance, three or more different drugs are used together to treat an infected individual. This is known as combination anti-retroviral therapy (cART) or highly active anti-retroviral therapy (HAART) (46–49).

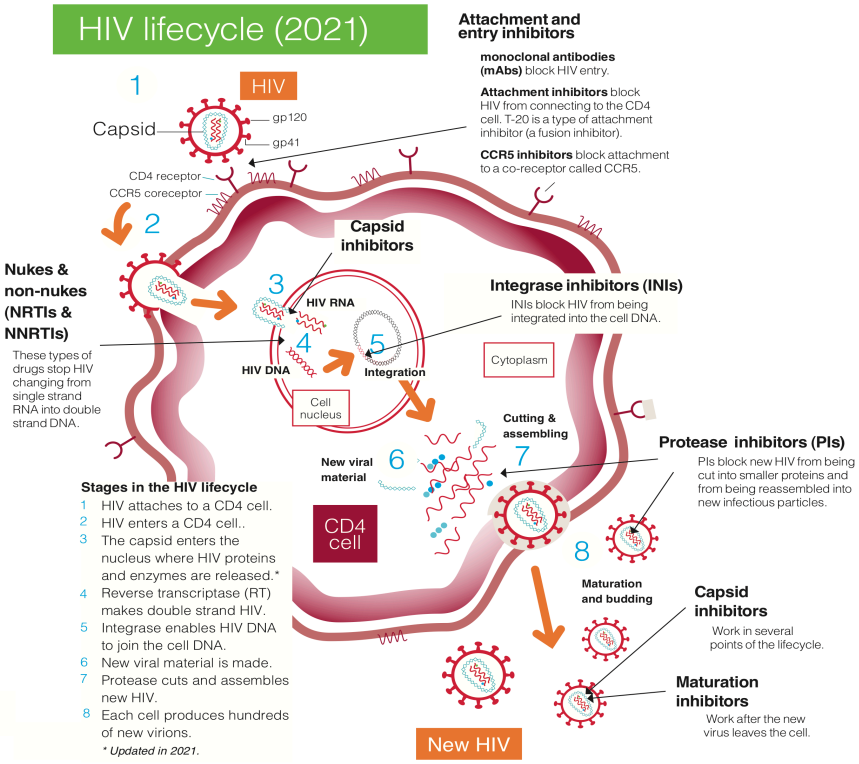


Figure 1.6 – HIV-1 antiretroviral drugs and target stages of HIV-1 life cycle. The various available classes of antiretroviral drugs inhibit different stages of the HIV-1 life cycle, resulting in a reduction in HIV-1 viral load. Source: i-Base.info (https://i-base.info/ttfa/section-3/5-how-hiv-drugs-work-main-types-of-drugs/).

While ART has enabled people living with HIV-1 to live long and relatively healthy lives, several disadvantages remain. These include complicated regimens leading to non-compliance, the need for lifelong treatment, financial burden, ART resistance and ART side effects including, but not limited to, gastrointestinal issues, central nervous system problems and haematological disturbances (50). The ever-increasing number of patients requiring lifelong treatment also places significant strains on health care systems, especially in developing countries with the highest burden of HIV-1 (51).

The need for lifelong ART in people living with HIV-1 is due to the fact that ART does not fully eradicate HIV-1 in infected individuals. Latent viral reservoirs, capable of being activated to produce infectious viruses, persist in long-living memory CD4+ T cells and immune cells in the central nervous system (CNS) that contain integrated provirus within host cellular DNA (52–55). Because these reservoirs are hidden from the immune system, they remain unaffected by ART.

According to UNAIDS, in eastern and southern Africa, there were an estimated 16.2 million people on ART in 2021. This is more than a three-fold increase from 2010 and represents 67% of all people living with HIV in southern and east Africa (http://aidsinfo.unaids.org). However, with 670 000 new transmissions in 2021 (http://aidsinfo.unaids.org), there is critical need for research into preventative and therapeutic treatments including vaccines for HIV-1.

1.6 Natural control of HIV-1

Individuals living with HIV-1 demonstrate significant variation with regards to rate of disease progression, with some individuals showing rapid disease progression while others remain asymptomatic for many years. HIV-1 controllers are a small subset of people living with HIV-1 who have a slow disease progression without the use of antiretroviral therapy (ART) (56). In our study, we have defined HIV-1 controllers as individuals who maintain a CD4+ T cell count greater than 500 cells/µl of blood without ART. HIV-1 controllers can be subdivided into a number of subgroups which include elite controllers (ECs; less than 1% of people living with HIV-1 in previously studied cohorts (57)) who are generally defined as having undetectable viral loads (VL; <50 RNA copies/ml), viraemic controllers (VCs), generally defined as having low detectable viral loads (50<VL<2000 RNA copies/ml), and high viral load long-term non-progressors (HVL LTNPs) who remain healthy for a minimum of 7 years without ART with viral loads exceeding 10 000 RNA copies/ml.

The viral load set point (VLS) is a good predictor of disease progression (Figure 1.7) (58–60). After initial acquisition, the level of HIV-1 viral RNA peaks, with a subsequent decrease until a VLS is reached, where viral RNA loads are maintained at a fairly constant level for months to years (61). Individuals with a higher VLS are more likely to have a faster disease progression to AIDS and consequently death (62,63). Multiple factors, both viral and immunological, have been proposed to have an influence on an individual's VLS.

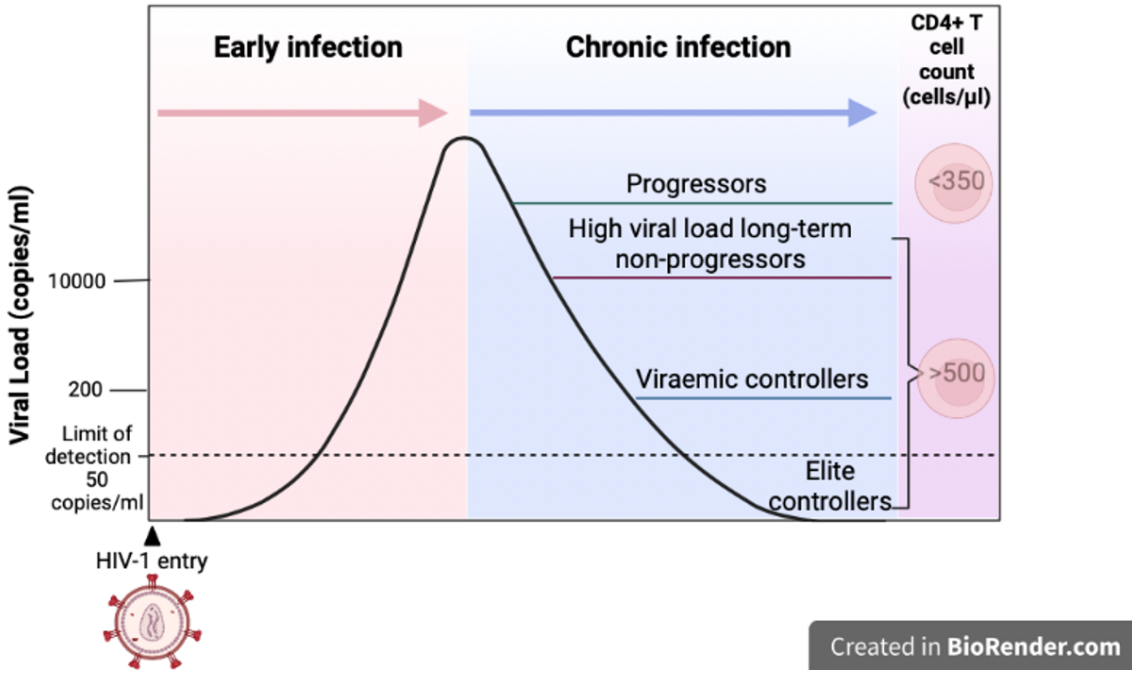


Figure 1.7 – The categories of individuals with differing rates of HIV-1 disease progression, based on viral load (RNA copies/ml) and/or CD4+ T cell count (cells/µl). HIV-1 controllers (elite controllers, viraemic controllers and high viral load long-term non-progressors) maintain healthy CD4+ T cell counts while progressors experience a marked decline in CD4+ T cell counts along with high HIV-1 viral loads.

HIV viral load levels, however, do not completely explain the rate of CD4+ T cell decline (64,65). Some individuals maintain low levels of viraemia but still have significant decreases in their CD4+ T cells and even experience AIDS-related events (66). There are also HIV-1 controllers who do not suppress viral load, yet maintain normal CD4+ T cell counts and do not experience AIDS-related symptoms. These interesting individuals exhibit low immune activation, a similar phenotype to that of SIV-infected sooty mangabeys and African green monkeys, who have high viral loads yet show no AIDS-related events (67). Interestingly, there was a report of an individual living with HIV-1

who had a viral load of >150 000 RNA copies/ml yet maintained normal CD4+ T cell counts without the use of ART for over 10 years (57).

1.7 Models of HIV-1 cure and mechanisms of control

HIV-1 cure encompasses both remission and eradication (68). While eradication refers to the complete elimination of intact and rebound-competent HIV-1 from an individual's cells, durable control is where viral load is undetectable and immune function is retained, mimicking when an individual is on effective ART (69). This has been reviewed in detail in Deeks et al., 2021 (68).

HIV-1 controllers thus represent an important model of HIV-1 durable control. Since different mechanisms of control are likely occurring in the different HIV-1 controller subgroups, the grouping and studying of all controllers as one group may limit the identification of factors that might be of importance. Further study into how HVL LTNPs and other individuals classified as HIV-1 controllers naturally control HIV-1 in the presence or absence of viraemia is essential to determining the correlates of protective immunity, which are important for the rational design of vaccines and novel therapies.

Intrinsic variability in both HIV-1 and the individual living with HIV-1 contributes to the varying levels of HIV-1 control (70).

1.7.1 HIV-1 virological fitness

The different HIV-1 subtypes vary in their pathogenicity, viral fitness, and chemokine coreceptor usage (71). In Thailand, a higher rate of transmission of HIV-1 subtype E viruses compared to subtype B viruses was observed (72), while in Uganda, there was a significantly higher rate of transmission with subtype A viruses compared to subtype D viruses (73).

The presence of HIV-1 variants using the CXCR4 coreceptor is associated with an increase in the rate of decline of CD4+ T cells, accelerated disease progression, and a poor prognosis for survival, as well as delayed viral suppression if present before starting ART (74). Studies have shown a low frequency of CXCR4 usage with subtype C strains [63], and an increased presence of CXCR4-using strains for subtype D HIV-1 [67]. In Uganda, a faster rate of disease progression was associated with infection with subtype D compared to subtype A (75). This could be attributed to the preferential CXCR4 usage in subtype D.

A higher degree of DNA methylation in the HIV 5′-long terminal repeat (LTR) was found in LTNPs and elite controllers compared to progressors which positively correlated with time of acquisition, indicating that HIV LTR silencing might be playing a role in reducing the number of replicating viruses resulting in a delayed progression of disease (76). While it has been hypothesised that defective viruses may be responsible for the low viral loads sustained by controllers, in the vast majority of cases the viruses isolated from HIV-1 controllers have been replication-competent, which suggests that host-mediated control is the main mechanism of viral suppression (77).

1.7.2 Host factors

There are various host factors that have been attributed to the ability of HIV-1 controllers to suppress viral replication (70,78–80). However, only a portion of previously studied HIV-1 controllers have an identified protective characteristic (viral and/or host) (81), suggesting that there are other, as yet undiscovered, factors involved. A variety of genes and gene polymorphisms that have either a positive or negative effect on infection and disease progression have been identified, including genes encoding for cytokines, chemokines, chemokine receptors like CCR2 and CCR5, HLA and intracellular host proteins like tripartite motif-containing protein 5α (TRIM5α) (82–84). Polymorphisms in *HLA* and in the *CCR2-CCR5* locus have been shown in select cohorts to account for approximately 25% of the observed variability in viral load (85–87).

Most of the research into mechanisms of control has focused on elite controllers and viral suppression, but less is known about the mechanisms of control exhibited by viraemic individuals (viraemic controllers and HVL LTNPs) where virus is still present but CD4+ T cell count is maintained.

1.8 Chemokines and HIV-1 control

1.8.1 CCR2

CCR2 is a major receptor for the ligand CCL2, a monocyte chemoattractant protein that mediates monocyte chemotaxis. CCR2 and CCL2 expression can be induced by HIV infection and/or the exposure to viral products, as evidenced by the high levels of CCL2 and CCR2 found in individuals living with HIV-1 (88). The CCL2/CCR2 axis is strongly connected to the increased level of immune activation and inflammation that is the hallmark of HIV, even in patients on ART (88).

CCR2 is an important genetic factor associated with resistance to HIV-1 infection. A mutation in the *CCR2* gene that causes a conservative amino acid change (isoleucine for valine) at position 64 (*CCR2-V64I*) in the first transmembrane domain of CCR2, significantly correlates with a delayed progression to AIDS (89). It is therefore important to evaluate polymorphisms in chemokine ligands and receptors as they may have an impact on HIV-1 transmission and disease progression through mechanisms beyond viral entry.

The CCR2 and CCR5 genes share significant sequence homology (73%), likely due to a gene duplication event (86). Multiple polymorphisms in both CCR2 and CCR5 are associated with HIV-1 control. Several variants in CCR2 and CCR5 exhibit linkage (87) and thus it is unclear which variant in which gene is conferring the functional effect on HIV-1 progression or acquisition. Population-specific patterns of CCR2 and CCR5 haplotypes might explain disparities in disease progression (87).

1.8.2 CCR5

CCR5, found on the surface of white blood cells, links the innate and the adaptive immune systems (90). It plays an important role in the inflammatory immune response by directing cells to sites of inflammation. CCR5 is the major coreceptor used by macrophage (M)-tropic and T-tropic strains of HIV-1 (91) and is generally required by HIV-1 in order to facilitate infection.

1.8.2.1 CCR5 ligands

There are multiple ligands of CCR5, of which CCL3, CCL4, CCL5 and CCL8 demonstrate the most suppressive activities in assays evaluating HIV-1 infection (92). While CCL3 and CCL5 are able to bind to other chemokine receptors, CCL4 is recognised as the most specific chemokine for CCR5. CCL3, CCL4 and CCL5 inhibit replication of R5 HIV-1 variants at the point of viral entry (93,94). Further, significantly higher levels of CCL3 and CCL4 are produced by CD8+ T cells in asymptomatic people living with HIV-1 compared to individuals that progress to AIDS and to individuals not living with HIV-1 (93).

1.8.2.2 CCR5 expression

Low CCR5 expression levels correlate with a reduced ability to infect T cells with macrophage-tropic HIV-1, *in vitro* (95). Expression levels of CCR5 affect numerous aspects of HIV-1

pathogenesis and outcomes of disease, with low CCR5 expression being protective, as individuals with lower expression levels of CCR5 have a lower immune activation (96–99). CCR5 is mainly expressed in memory T cells, macrophages, and immature dendritic cells, and is upregulated by pro-inflammatory cytokines (91,100,101). The differential expression of CCR5 may in part be explained by polymorphisms in the *cis*-regulatory regions of *CCR5.* The density of coreceptors (i.e., the mean number of coreceptors at the surface of cells) also contributes to HIV-1 progression in various ways (98,102–104). Increased CCR5 density at the surface of CD4+ T cells and central memory CD4+ T cells was shown to associate with higher viral loads and faster disease progression in HIV-1-infected persons and had an effect on the post-entry efficiency of R5 HIV-1 infection (104,105).

1.8.2.3 CCR5 genetic polymorphisms

There are many *CCR5* polymorphisms associated with HIV-1 control. One of the most studied genetic variants implicated in HIV-1 control is the Δ32 mutation in the *CCR5* gene (*CCR5Δ32*). *CCR5Δ32* in the homozygous form renders the CCR5 coreceptor non-functional and CCR5 is not expressed on the cell surface due to a 32 bp deletion in the gene sequence which introduces a premature stop codon (70). Individuals homozygous for the mutation tend to be resistant to HIV-1 R5 viruses because they require the CCR5 coreceptor to gain entry to CD4+ T cells and macrophages. People living with HIV-1 that are heterozygous for the *CCR5Δ32* polymorphism have a significantly slower disease progression (106,107). *CCR5Δ32* is predominantly found in populations of European descent and is virtually absent in African, East Asian and American Indian populations (108), emphasizing the importance of population-specific studies.

There are also nine well-studied *CCR5* promoter haplotypes (HHA, HHB, HHC, HHD, HHE, HHF*1, HHF*2, HHG*1, HHG*2) affecting CCR5 expression levels, that have been shown to have a varying effect on HIV-1 disease progression (109). These haplotypes are defined by seven *CCR5* 5'UTR SNPs and the presence/absence of *CCR5Δ32* and *CCR2-V64I* (110,111) (Figure 1.8).

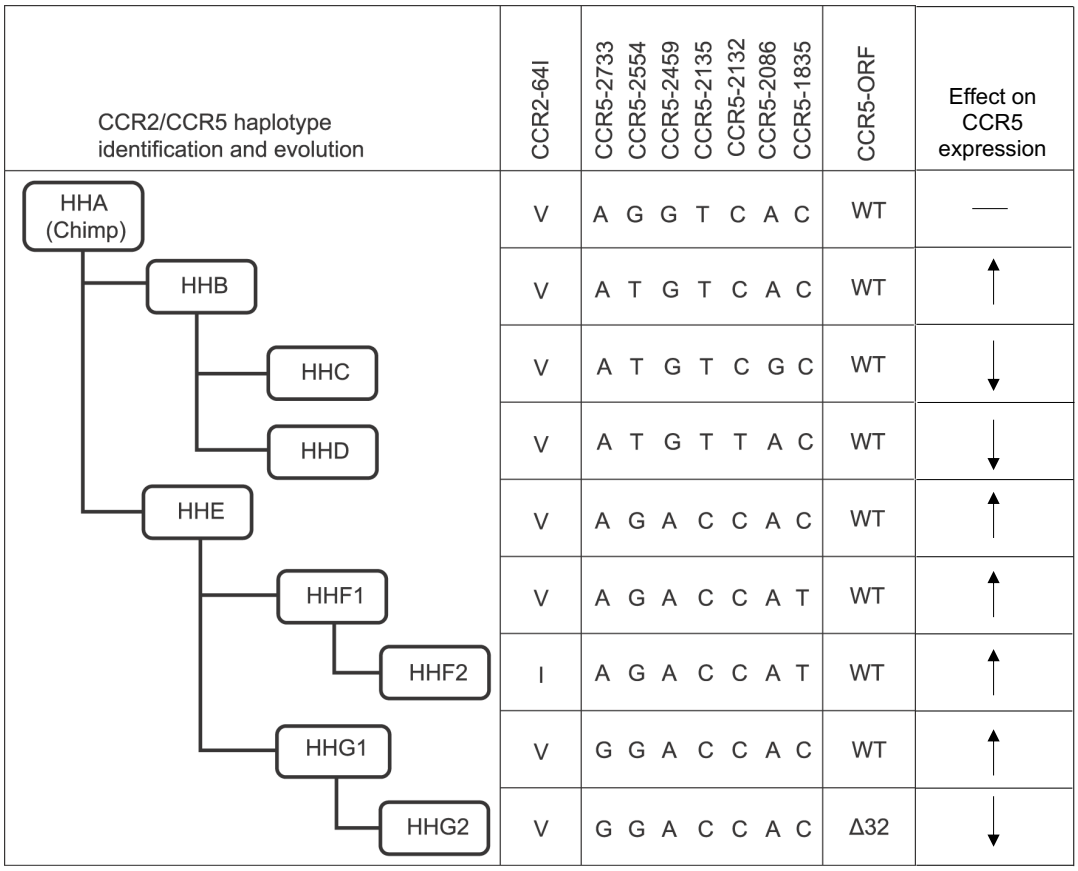


Figure 1.8 - Polymorphisms defining the nine *CCR5* haplotypes. Haplotypes are defined based on the evolution of linked CCR2 and CCR5 mutations, including *CCR2-V64I* and the *CCR5* -2733 A>G, -2554 G>T, -2459 G>A, -2135 C>T, -2132 C>T, -2086 A>G and -1835 C>T polymorphisms, and the delta-32 deletion in the open reading frame of *CCR5 (CCR5-ORF).* Adapted from (411).

The haplotypes have different promoter activities, correlating with differential cell surface CCR5 expression (109,112–115) and affect rates of HIV-1 disease progression and HIV-1 acquisition very differently depending on the population studied (116). The protective effect of *CCR5* promoter haplotypes with respect to HIV-1 control may be associated with this reduction in cell surface CCR5 levels.

Individual SNPs in the *CCR5* promoter region have been shown to affect CCR5 expression levels. The −2459 G>A SNP (rs1799987) is in complete linkage disequilibrium (LD) with the -2135 T>C SNP (rs1799988) and is common to the HHE, HHF and HHG haplotypes. The −2459 G>A SNP (and therefore the -2135 T>C SNP by association) has been associated with the rate of progression to AIDS, where individuals with the GG genotype exhibit slower disease progression than those

with the AA genotype (117–119). The -1835 C>T SNP (found in the HHF haplotypes) is an intronic SNP and is in complete LD with the *CCR2-V64I* polymorphism (107,120). Possession of these two polymorphisms was found to be protective with regards to HIV-1 disease progression (120).

Genome wide studies have shown a strong association with HIV-1 outcomes for the rs1015164 (G>A) polymorphism. This SNP has been shown to mark expression of the antisense long non-coding RNA (lncNRA) CCR5AS (121). CCR5AS expression levels in CD4+ T cells significantly positively correlated with levels of CCR5 mRNA expression (r=0.42, p=0.0001) (121). The rs1015164 SNP had a genome-wide effect independent of other SNPs in the region, including *CCR5Δ32* ($p = 1.5\times10-19$) (121). The DNA methylation status of *cis*-regions in *CCR5*, specifically in intron 2, has also been implicated in HIV-1 control, inversely correlating with CCR5 levels on T cells (115). The rs553615728 -4223 C>T single nucleotide polymorphism (SNP) disrupts a cytidine phosphate guanidine (CpG) dinucleotide in the *cis*-region of *CCR5, namely* CpG-41, a binding site where DNA methylation occurs, and has been uniquely found in individuals from southern Africa (115).

1.8.3 CXCR6

CXCR6, also known as Bonzo and STRL33, has been identified as a minor coreceptor used by HIV-1 to enter target cells, in conjunction with CD4. While nearly all HIV-1 strains use CCR5 and/or CXCR4, most low-pathogenicity SIV strains use CXCR6 a major coreceptor (122–124). Expression of CXCR6 has been detected on a variety of cells including natural killer (NK) cells, dendritic cells, activated T cells and NKT cells (125,126). CXCR6 is a marker for effector T cells (127,128) and promotes homing of lymphocytes to extra-lymphoid tissue (129). CXCR6 and its ligand CXCL16 are thought to play an important role in T cell trafficking and cell to cell contact during inflammation (130).

NK cells in fetal liver and spleen expressing CXCR6 produced more cytokines and degranulated more robustly than CXCR6 negative NK cells (131). Interestingly, hepatic CXCR6 positive NK cells have also been shown to mediate antigen-specific memory responses against haptens, virally encoded proteins, and viruses (132). It was shown that CXCR6 positive NK cells upregulated TNF-related apoptosis-inducing ligand (TRAIL), a key death ligand in hepatitis pathogenesis and that a

subset of liver-resident NK cells, distinguished by their surface expression of CXCR6, were adapted for hepatic tolerance and inducible anti-viral immunity (133).

1.8.3.1 CXCR6 genetic polymorphisms

In a genome-wide association study (GWAS), the *CXCR6* rs2234358 polymorphism (G>T), located 42 base pairs (bp) downstream from the CXCR6 termination codon, was the variant found to be most significantly associated with HIV-1 control in viraemic individuals (i.e. viraemic controllers (non-elite controllers); $p=2.5 \times 10^{-7}$), independent of the CCR2-CCR5 locus (130), and this association was replicated in additional studies, resulting in a genome-wide significance of $p=9.7 \times 10^{-10}$ (134).

In a previous study from our research group investigating this SNP in natural HIV-1 control in black South Africans, the rs2234358 TT genotype was significantly underrepresented in viraemic controllers compared to both healthy controls and progressors ($p=0.001$, $p_{bonferroni}=0.036$ and $p=0.006$, respectively) (135). Interestingly, in the small HVL LTNP group (n=11), the rs2234358 T allele frequency was higher (72.7%) than in all other study groups, with no individuals in this group homozygous for the rs2234358 G allele. It has been postulated that the rs2234358 SNP may affect disease progression either by directly altering CXCR6 expression/function or through interaction/association with other SNPs in the *CXCR6* promoter region (135).

An additional SNP, rs2234355, found in the N-terminus of CXCR6 (CXCR6-E3K), has been associated with protection from *Pneumocystis carinii* pneumonia-mediated progression to death among African Americans living with HIV-1 (136), and with faster virological failure in Caucasian patients who had initial viral load suppression on ART (137). This SNP is highly prevalent in African Americans (44%) while being extremely rare in European populations (<1%), again emphasizing the importance of population specific studies.

Data from our research group showed that CXCR6-E3K SNP heterozygosity was significantly overrepresented in viraemic controllers when compared to healthy controls and progressors ($p=0.003$ and $p=3.8 \times 10^{-5}$, respectively) (135). The CXCR6 E3K mutation has been predicted to result in reduced surface expression of CXCR6 (138), suggesting that CXCR6 expression may be implicated in HIV-1 control and highlighting the importance of a better understanding of CXCR6 function.

When investigating the combinatorial effect of the absence of the deleterious rs2234358 TT genotype and the presence of advantageous rs2234355 heterozygosity (i.e. -358TT/+355GA), viraemic controllers had a highly significant overrepresentation of -358TT/+355GA compared to healthy controls and progressors (p=1x10^{-6}, $p_{bonferroni}$=3.4x10^{-5} and p=2x10^{-9}, $p_{bonferroni}$=6.8x10^{-8}, respectively), suggesting an additive effect of the presence of rs2234355 GA and absence of rs2234358-TT in natural control of HIV-1 in the presence of detectable viraemia (50>VL<2000 RNA copies/ml plasma) (135).

1.9 Regulation of gene expression

Many polymorphisms in CCR5 and CXCR6 that have been associated with HIV-1 control affect expression levels of these proteins. Regulation of a gene occurs at all levels during the process of converting DNA into a protein product. The core promoter is generally defined to be the DNA region that directs the initiation of transcription by RNA polymerase II (139). Regulatory elements are contained within the promoter sequence and at the transcription start site of a gene (140). A regulatory sequence acts to modulate the expression of genes (140). Promoter usage has a major impact on gene expression, with many mammalian genes containing multiple promoters (141). Alternative promoter use is a common occurrence in humans which can alter expression of the associated gene at both the mRNA and protein level (141).

In addition, regulation occurs after transcription in the 3' untranslated region (3'UTR) of the mRNA, resulting in differing mRNA stability and affecting translation of the mRNA into a protein (142). The 3′UTR, situated downstream of the protein coding sequence, has been found to be involved in numerous regulatory processes including transcript cleavage, stability and polyadenylation, translation and mRNA localization. These regions are thus critical in determining the fate of an mRNA (143). Regulatory elements within the 3'UTR are mostly expected to function post-transcriptionally at the mRNA level, but they can also function at the DNA level as distal enhancers to control transcription (144). To our knowledge, no detailed characterization of the 3'UTR of *CCR5* and *CXCR6* in the context of HIV-1 control in the presence of viraemia has been reported.

1.10 HIV-2 and SIVsmm as models of durable control

1.10.1 HIV-2

Human immunodeficiency virus type 2 (HIV-2) is less pathogenic than HIV-1 and is mostly confined to West Africa. People living with HIV-2 generally have a longer asymptomatic stage than people living with HIV-1 (141). HIV-2 has a lower transmissibility and a slower progression to AIDS (145). A summary of differences between HIV-1 and HIV-2 can be seen in Table 1.1.

HIV-2 shares 30–40% homology with HIV-1 *env* sequences and 60% homology with HIV-1 in the *gag* and *pol* sequences (146). In a study of infection rates of peripheral blood cells in 14 untreated individuals living with HIV-2, results showed that HIV-2 had a poor replicative capacity, did not infect monocytes, and preferentially infected transitional memory CD4+ T cells, with very low infection in central memory CD4+ T cells (147).

HIV-2 is capable of using a broader range of coreceptors to enter target cells than HIV-1 and is able to efficiently use CCR5, GPR15 and CXCR6 (148). Interestingly, in a group of HIV-2 infected individuals, CXCR6 and GPR15 were used more frequently in aviraemic individuals than in viraemic individuals (148). In LTNPs living with HIV-1 who possess protective HLA-B*27 or B*57 alleles, central memory CD4+ T cells were found to have a significantly lower infectivity level than transitional memory CD4+ T cells (149). The preservation of long-lived CD4+ central memory T cells is integral for long-term immunological memory (150–152). In HIV-2 infected individuals, transcriptome analysis showed an increased expression of CXCR6 genes in transitional memory CD4+ T cells compared to central memory CD4+ T cells ($p=0.023$) and low circulating HIV-2 reservoirs were mainly distributed in transitional memory CD4+ T cells (147).

Table 1.1 - Comparison of immune response to infection with HIV-1 and HIV-2.

Characteristic	HIV-1	HIV-2
Infectivity	High	Low
Virulence	High	Low
Prevalence	Global	West Africa
Origin	Common Chimpanzee	Sooty Mangabey
Clinical illness	Majority develop AIDS	20-25% develop AIDS; remainder are LTNPs
Plasma viral load	High in acute phase and during disease; may be undetectable in asymptomatic phase	High in progressors, undetectable in LTNPs
CD4+ T cell count	Decreases during acute infection, returns to normal and then declines over time	Normal in LTNPs, reduced count in progressors
CD4+ depletion in gut associated lymphoid tissue	Massive depletion	Unknown
Vertical transmission	Up to 40%	<4%
Immune activation	Elevated even when viral load is undetectable	Not elevated in LTNPs, increased in progressors and predicts disease progression
T cell proliferation	Increased CD4+ and CD8+ T cell turnover	Unknown
Thymopoiesis	Reduced	Enhanced (maintains CD4+ T cells)
T cell apoptosis	Increased	Lower than HIV-1
Virus-specific CD8+ T cell response	Vigorous. Gag-specific response correlates with reduced viral load	Vigorous. Magnitude and Gag peptide specificity inversely correlate to viral load
Selection of cytotoxic T cell escape variants	Frequently occurs; associated with clinical decline	Unknown
Virus-specific CD4+ T cell response	IFNγ response present throughout infection. IL-2 secretion and proliferation correlate with LTNP status	IFNγ response present in most patients. IL-2 secretion and proliferation correlate with LTNP status

Adapted from (412,413). LTNP: long-term non-progressor, IFNγ: intereferon gamma, IL-2: interleukin 2.

1.10.2 SIV

1.10.2.1 Natural versus non-natural hosts

A key feature of SIV in natural hosts (i.e., sooty mangabeys (SMs) and African green monkeys (AGMs)) is the lack of clinical disease (67). However, experimental SIV transmission to non–natural host Asian monkey species (i.e., rhesus macaques (RMs)) results in the development of illness similar to human AIDS (153).

Similarities between SIV of natural hosts and pathogenic HIV/SIV transmissions of humans and RMs include the presence of high viremia, a short lifespan of productively infected cells, a substantial loss of mucosal CD4+ T cells during acute infection, high levels of immune activation during acute infection and the inability to control virus replication (reviewed by (154)).

Unique features of non-pathogenic SIV include an absence of disease progression in the presence of high viral loads and high viral replication rates in the intestine, fast healing of virally-induced inflammation, a lack of microbial translocation, preservation of healthy levels of peripheral CD4+ T cells (155), preferential sparing of central memory CD4+ T cells from direct virus infection (156,157), a lack of chronic immune activation, rapid control of viral replication in the secondary lymphoid organs and a lack of viral trapping in follicles (reviewed by (154)). While the depletion of effector memory CD4+ T cells was shown to be the immediate mechanism behind immunodeficiency, SIV disease progression was found to be largely determined by the destruction, failing production, and gradual decline of central memory CD4+ cells (158).

A summary of how disease progression in natural SIV hosts differs from pathogenic HIV/SIV in humans and rhesus macaques is shown in Table 1.2.

Table 1.2 - Main features of SIV infection of natural hosts compared to non-natural hosts.

	Natural Host (Sooty mangabeys/African green monkeys)	**Non-Natural Host (Rhesus macaques/ humans)**
AIDS	**No**	Yes
Level of peripheral CD4+ T cells	**Healthy**	Low
Viral load	High	High
Virus cytopathicity	Yes	Yes
Host immune control	Ineffective	Ineffective
Depletion of mucosal CD4+ T cells	Yes, stable	Yes, progressive
Mucosal immune dysfunction /microbial translocation	**No**	Yes
Chronic immune activation	**No**	Yes
Pattern of infected cells	**Tem > Tcm**	Tcm > Tem
Vertical transmission	**Rare**	Frequent

Differences in natural hosts compared to non-natural hosts are bolded. Tem: effector memory T cells, Tcm: central memory T cells. Adapted from (154).

1.10.2.2 SIV preferential coreceptor-usage

While CXCR6 is considered a minor coreceptor for HIV-1, it has been shown that CXCR6 is a major coreceptor in some simian immunodeficiency virus (SIV) hosts, such as sooty mangabeys (122,159). It is yet to be shown if the use of the CXCR6 coreceptor by multiple primate species was selected for its ability to allow viral replication in an environment where CCR5 may be limited, or whether this occurred as a consequence of selection for different factors that the CXCR6 gene may be linked to (123). It has been proposed that this preferential use of CXCR6 for entry by SIV, in addition to low CCR5 levels in these hosts, may result in virus being redirected to different cell targets than hosts with CCR5 as the main receptor, and that this differential targeting may promote infection of non-essential cells and limit infection of critical cells (122).

The SIV-1 that macaques acquire (SIVmac; macaques go on to develop AIDS) cannot use species-matched CXCR6 due to a single amino acid substitution (S31R) in the N-terminus of macaque CXCR6 (122,160), while the SIV-1 that sooty mangabeys acquire (SIVsmm; sooty mangabeys maintain healthy CD4+ T cells counts in the presence of very high viraemia and do not develop AIDS) primarily uses CXCR6 (122).

In sooty mangabey lymphocytes, CXCR6 was found to be restricted to CD4+ effector memory T cells and was expressed by a distinct sub-population to those expressing CCR5 (123). In sooty mangabeys with SIV, central memory CD4+ T cell reservoirs are limited, thought to be related to low CCR5 coreceptor expression (156). Lack of infection of central memory CD4+ T cells seems to play a role in low pathogenicity models of both HIV and SIV (156,161).

While SIVmac cannot use CXCR6 due to a polymorphism in macaque CXCR6, the reason HIV-1 rarely uses CXCR6 is due to the viral Env itself (159,162). A study examining the effect of the loss of CXCR6 coreceptor usage in the setting of HIV-1 and SIV showed that coreceptor sensitivity was determined by the HIV-1 *env* gene, with Pro326 in the third variable region (V3) crown abrogating CXCR6 use (123). This amino acid was found to be absent in monkey SIVs but highly conserved in SIV of wild chimpanzees (SIVcpz) and HIV-1 (123). Amino acid diversity in the glycoproteins within Env have been shown to have up to 35% variation between HIV-1 subtypes and up to 20% variation within the same subtype (163), resulting in small but perhaps significant structural differences in this protein across the various HIV-1 subtypes (39,164).

Interestingly, an HIV-1 controller homozygous for *CCR5Δ32* was shown to possess virus that was able to engage alternative viral coreceptors, including CXCR6, *in vitro*.

1.11 Study rationale

Little is known about the mechanisms of control employed by HVL LTNPs or individuals that maintain stable CD4+ T cell counts in the presence of viraemia. A complete understanding of the different molecules that HIV uses to enter immune cells is necessary in order to develop effective strategies to inhibit the virus at different stages of infection.

The chemokine receptor CCR5 is an integral molecule with regards to both the acquisition and pathogenesis of HIV-1. CCR5 expression increases with HIV-1 disease progression and decreases after the initiation of antiretroviral therapy, further emphasizing its importance with regards to HIV-1. Describing haplotypes and polymorphisms in *CCR5* provides necessary and important baseline knowledge that could contribute to the understanding of differential expression of this coreceptor in a South African context.

The distinct differences in pathogenicity between HIV-1 and SIVsmm have provided a unique opportunity to look for protective viral and host immune mechanisms that contribute to viral control. The preferential usage of the CXCR6 coreceptor over the CCR5 coreceptor by SIVsmm results in a lower pathogenicity of the virus. The fact that CXCR6 polymorphisms have been associated with HIV-1 control in viraemic individuals suggests that CXCR6 may be of importance in these specific controllers.

To our knowledge, little is known about the constitutive background expression of CCR5 and CXCR6 in a South African context and how known polymorphisms associate with variations in expression levels. Previous studies investigating CCR5 and CXCR6 with regards to HIV-1 disease have been mainly conducted in cohorts of European descent (i.e., Caucasian). Although the burden of HIV-1 is highest in sub-Saharan Africa, people living with HIV-1 in this region remain understudied compared with populations living with HIV-1 in Europe and the United States of America. Apart from remarkable differences in host genetic backgrounds, populations from these different geographical regions are exposed to different HIV subtypes, with sub-Saharan populations predominantly acquiring HIV-1 subtype C.

1.12 Aim of study

In summary, the overall aim of this study was to explore coreceptor related factors contributing to HIV-1 control. We investigated genetic variants in *CCR5* and HIV-1 control in people living with HIV-1 (controllers and progressors), and assessed CCR5 and CXCR6 constitutive expression (frequency and density) and the potential association with genetic polymorphisms in individuals without HIV-1. The effect of ethnicity, cell activation, biological sex and age on these findings is also described.

1.13 Specific objectives

i. To describe genetic variation in the regulatory regions of *CCR5* and to investigate the role of discovered *CCR5* variants on HIV-1 control
ii. To quantitate CCR5 and CXCR6 receptor expression in whole blood of South African healthy controls to describe constitutive expression on different cell types
iii. To assess the effects of ethnicity and immune activation on CCR5 and CXCR6 expression levels
iv. To assess the effects of sex and age on CCR5 and CXCR6 expression levels
v. To investigate the effect of *CCR5* and *CXCR6* variants on cell surface expression of the respective coreceptors

CHAPTER TWO

2. Materials and Methods

2.1 Sample population

2.1.1 People living with HIV-1

Participants included black South African ART-naïve HIV-1 controllers which comprised of 23 elite controllers (ECs), 37 viraemic controllers (VCs) and 11 high viral load long-term non-progressors (HVL LTNPs). This study also included 74 ART-naïve progressors who required initiation of ART upon enrolment. Cohort characteristics are described in Table 2.1.

Table 2.1 - Characteristics of study cohort of people living with HIV-1.

Group	Number of participants (n)	Age [years] (Mean and range)	Gender [% female]	CD4+ T cell count* [cells/µl] (Median and IQR)	Viral load* [HIV RNA copies/ml] (Median and IQR)	Years since diagnosis (Median and IQR)
ECs	23	48 (27-66)	78.2	693 (588 - 969)	<20	15 (8-16)
VCs	37	42 (27-59)	91.9	704 (587 - 910)	598 (135 – 1180)	8 (8-15)
HVL LTNPs	11	43 (33-53)	81.8	660 (606 – 749)	22 410 (14262 – 77820)	8 (8–11)
Progressors	74	45 (30–73)	83.8	177 (145 – 210)	38 444 (19790 – 103314)	6 (1–7)

*CD4+ T cell counts and viral loads of controllers used were from time of enrolment whereas for the progressors the last CD4+ T cell count and viral load prior to ART initiation was used. ECs: elite controllers, VCs: viraemic controllers, HVL LTNPs: high viral load long-term non-progressors.

Viral loads (RNA copies/ml of plasma) were quantitated using the COBAS®AmpliPrep/COBAS®Taqman® HIV-1 Test, v2.0 ultrasensitive tests (<20 RNA copies/ml) (Roche Diagnostic Systems, Inc, New Jersey, USA) and CD4+ T cell counts (cells/µl of blood) were determined using the FACSCount™ System (Becton Dickinson, San Jose, California, USA).

2.1.2 Healthy controls

Healthy black (n=17) and white (n=21) South African individuals not living with HIV-1 were prospectively recruited from the National Institute for Communicable Diseases campus, Gauteng, South Africa, as healthy controls. Individuals were born in South Africa and self-classified as either black or white. Controls were age- and sex-matched. Black South Africans had a median age of 31 years (IQR: 28-43 years) with 52.9% female representation and white South Africans had a median age of 35 years (IQR: 31-49 years) with 52.4% female representation.

2.1.3 Ethics

Written informed consent was obtained from all individuals participating in this study and ethics approval (Certificate number M190995; Appendix A) was obtained from the Human Research Ethics Committee at the University of the Witwatersrand, Johannesburg, South Africa.

2.2 Standard polymerase chain reaction (PCR) amplification of *CCR5* 5'UTR and 3'UTR

Genomic DNA was extracted from either whole blood or buffy coats of patients using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Each region of interest was PCR amplified with primers designed using PrimerW software using the EXPAND High Fidelity PCR System (Roche, Mannheim, Germany) with cycling conditions carried out according to manufacturer's instructions. The mastermix used for all PCR reactions contained a 300 nM final concentration of each primer, 200 µM final concentration of dNTPs and 1.5 mM final concentration of $MgCl_2$. The resulting PCR products were electrophoresed on 1% agarose gels for larger sized products (>500 bp) and 2% agarose gels for smaller fragments (<500 bp). Fermentas Middle Range Molecular Weight markers (Thermo Fisher Scientific, Massachusettes, USA) were used for size referencing. The gels were run for 30-40 minutes at 100V. The PCR amplified products were then purified using either the MSB Spin PCRapace kit (STRATEC Molecular, Berlin-Buch, Germany) or Agencourt Ampure XP (Beckman Coulter, Missouri, USA) magnetic separation kit according to manufacturer's instructions, in preparation for downstream applications.

2.3 Sequencing of CCR5 5'UTR and 3'UTR

Sequencing reactions were set up in 96-well plates using BigDye Terminator version 3.1 Cycle Sequencing Chemistries (Applied Biosystems, Foster City, CA, USA). Purified PCR amplicons were used as the DNA template. After cycling, the sequencing reactions were purified using ethanol-sodium acetate precipitation. Briefly, 35 µl of an ethanol-sodium acetate solution was added to the sequencing reactions (10 µl) and the plate was centrifuged on a Jouan B4i Multifunction Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 448g for 30 minutes. The plate was then placed upside down on absorbent paper and centrifuged at 3g for 1 minute. Subsequently, 70% ethanol (50 µl) was added to each well and the plate was centrifuged at 448g for 5 minutes. Again, the plate was placed upside down in the same manner as described above and centrifuged at 3g for 1 minute. The plate was then dried for 3 minutes at 63°C on a GeneAMP PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Dried sequencing pellets were resuspended in 10 µl of HiDi Formamide (Life Technologies, California, USA). The resuspended sequenced fragments were subsequently heated at 95°C for 2 minutes and then electrophoresed on an automated 3100 Genetic Analyser (Applied Biosystems) using Pop6 polymer and a 36cm capillary array (Life Technologies, California, USA), according to manufacturer's instructions. The PCR and sequencing primers used are shown in Table 2.2.

Table 2.2 - Primers used for PCR and sequencing of CCR5 5'UTR and 3'UTR regions.

Region	Application	Primer	Primer sequence (5' – 3')
CCR5 5'UTR	PCR	Forward primer	CCAAGCACCAGCAATTAGC
		Reverse primer	TGCCACCACAGATGAATGTC
	Sequencing	Pd2	ATTCTAGAGCCAAGGTCACG
		Pd3	TCCTGCCACCTATGTATC
		Pd4	GGTTAATGTGAAGTCCAGG
		Pd5	CTAACAGATTCTGTGTAGTGG
		Pd6	GTAACCTCTCAGCTGCTTG
CCR5 3'UTR	PCR (Amplicon A)	Forward primer	CAGTAGCTCTAACAGGTTGGAC
		Reverse primer	AGATCTCATGTGTGACCTGAAG
	PCR (Amplicon B)	Forward primer	GAGATCCTGGTTGGTGTTGC
		Reverse primer	GTAAGTGACCAGGCCATGAC
	Sequencing	UTR-F2	CAGTAGCTCTAACAGGTTGGAC
		ORF-5	GACCCAGTCAGAGTTGTGC
		ORF-6	AACTCTCCCTTCACTCCG
		ORF-7	GAACCAGGCGAGAGACTTG
		UTR-1	CAAGGAGACCACCAACAGC
		UTR-2	TGCAGAGCTTGAACACAGTC
		UTR-3	TTCTATGAGGCAACCACAGG
		UTR-4	GCGCCTTAGGTACTTATTCC

2.4 Sequence analysis

Resulting sequence chromatograms were analysed using Sequencher software version 5.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Imported sequences were aligned to a reference sequence obtained from the NCBI database (NCBI ref seq NC_000006.12). The numbering of *CCR5* SNPs used in this study is as described by Mummidi et al., 2000 (109), with the first nucleotide of the translational start site designated as +1 and the nucleotide immediately upstream from that designated as −1.

2.5 CCR5 haplotype assignment

Individuals were assigned previously described *CCR5* haplotypes (HHA, HHB, HHC, HHD, HHE, HHF*1, HHF*2, HHG*1, HHG*2) based on the presence or absence of seven 5'UTR SNPs at positions −2733 (rs2856758), −2554 (rs2734648), −2459 (rs1799987), −2135 (rs1799988), −2132

(rs41469351), −2086 (rs1800023) and −1835 (rs1800024), and the presence/absence of *CCR2-V64I* (rs1799864) and *CCR5Δ32* (rs333). The polymorphisms defining each haplotype are shown in Figure 2.1.

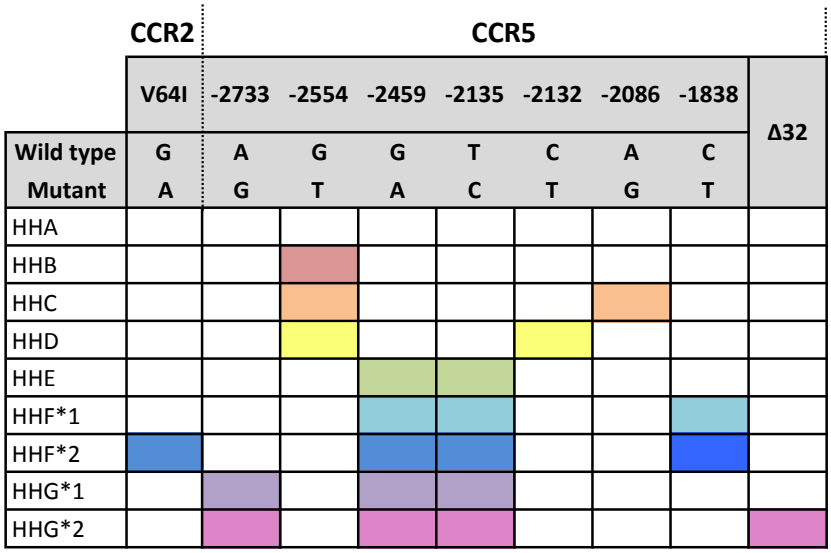


Figure 2.1 - Polymorphisms forming each CCR5 promoter haplotype. The *V641* variant is located in the *CCR2* gene, while the other variants are located in the *CCR5* gene. Colour shaded boxes show the respective SNPs or indels that form each haplotype.

2.6 Single nucleotide polymorphism (SNP) genotyping

Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. For single nucleotide polymorphism (SNP) genotyping, cycle threshold (C_q) qPCR assays were utilised, except for the +2919 T>G SNP where a predesigned TaqMan™ SNP Genotyping assay was used as described below. For parts of the study involving people living with HIV-1, individuals were genotyped for the presence/absence of *CCR2-V64I* (rs1799864), the *CCR5* -4223 C>T (rs553615728) SNP and *CCR5Δ32* (rs333). Healthy controls were genotyped for variants that have been previously associated with HIV-1 control, namely the *CCR2-V64I* (rs1799864), *CCR5* -4223 C>T (rs553615728), -2459 G>A (rs1799987), rs1015164 G>A, +2919 T>G (rs746492) SNPs and *CCR5Δ32* (rs333), and the *CXCR6* rs2234355 G>A and rs2234358 G>T SNPs.

A predesigned TaqMan™ SNP Genotyping assay was used to genotype individuals for the *CCR5* +2919 T>G SNP (Applied Biosystems, Foster City, CA, USA; catalog number 4351379), according to manufacturer's instructions. This assay utilises TaqMan 5′-nuclease chemistry to amplify and detect polymorphisms. Each assay consists of two sequence-specific primers and two TaqMan minor groove binder (MGB) probes with non-fluorescent quenchers (NFQ). One probe is labeled with VIC dye to detect the wild-type sequence and the second probe is labeled with FAM dye to detect the minor allele sequence.

For all other genotyping, cycle threshold (C_q) qPCR assays were designed using SYBR Green to detect the SNP using allele-specific PCR, with two primers used; one specific for the major or wild-type (WT) allele and one specific for the minor or mutant (Mt) allele of each SNP, and one common primer in the forward or reverse orientation depending on the orientation of the allele-specific primers. Thus, for each sample, two reactions were conducted, one with the major allele (WT) primer and one with the minor allele (Mt) primer. The mispriming of 3' end primer nucleotides differentiated between the different genotypes. In order to develop the C_q shift assays, it is required to have individuals of known genotype at the SNP positions, determined by amplifying and sequencing the region harbouring the respective SNPs from control samples. The assays were optimised using two known homozygous WT individuals, two known heterozygous individuals and two known homozygous Mt individuals for each of the SNPs.

Reactions (final volume of 10 µl) were set up in 96-well plates, with each reaction containing 1× SYBR Green PCR Master Mix, 10 ρmol of each forward and reverse primer, and ~10-50 ng of genomic DNA as template. The reactions were then run in an Applied Biosystems 7500 Real-Time PCR system. A no template control (NTC) was included and a melt-curve analysis was performed to ensure the absence of primer-dimers. Run settings included a holding stage for 10 minutes at 95 °C and a cycling stage including a denaturation step of 95 °C for 15 seconds, an annealing step and an extension step. Annealing and extension settings were optimised for each SNP assay and are shown in Table 2.3, along with the primer sequences and SYBR Green reagent used. The primers were designed using PrimerW software, with some utilizing locked nucleic acid (LNA™) modified 3' end nucleotides. The *CCR2-V64I* and *CCR5Δ32* allelic discrimination assays were previously designed (101,165) and some individuals from our cohort were previously genotyped for the *CCR5*

-4223 C>T SNP (115). The remaining individuals were genotyped using a C_q qPCR assay as described above, that was designed using available heterozygotes from the prior genotyping.

Homozygous WT individuals had a resulting C_q shift (i.e., difference in cycle threshold between the WT reaction and Mt reaction) due to mispriming of the Mt primer in that respective well, heterozygous individuals resulted in both primers binding optimally, resulting in no C_q shift, while homozygous Mt individuals resulted in a shift similar to the homozygous WT individuals, but with the Mt primer binding optimally (Figure 2.2). Data were analysed using software supplied with the 7500 Real-Time PCR system and the genotype of each sample was recorded.

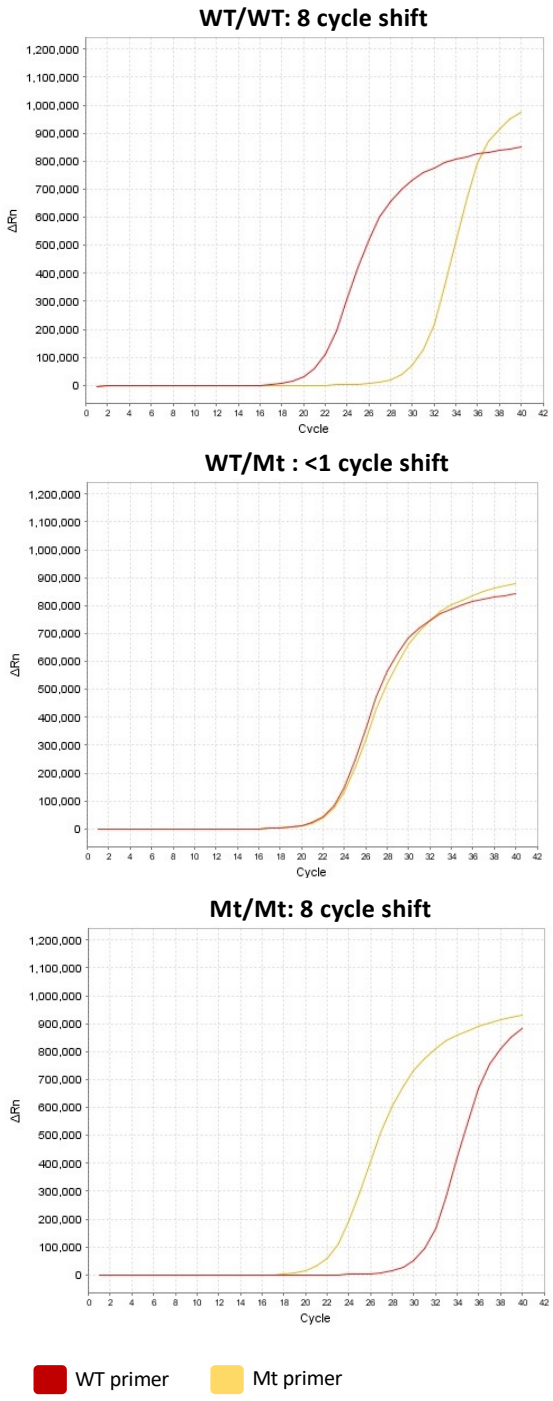


Figure 2.2 – Representative C_q shift amplification plots for a WT/WT, WT/Mt and Mt/Mt genotype. WT/WT represents both alleles being of the ancestral/wild-type, WT/Mt represents one allele being of the ancestral/wild-type and one allele being of the mutated type, and Mt/Mt represents both alleles being of the mutated type. Colours representing the WT primer and the Mt primer are shown at the bottom of the figure.

Table 2.3 – C_q qPCR assay primer sequences, run settings and SYBR Green reagent used.

Gene	SNP	Common primer (5'-3')	Allele-specific primers (5'-3')	SYBR Green	Annealing	Extension
CCR2	rs1799864	(F) AACGAGAGCGGTGAAGAAG	(R) TTTGCAGTTTATTAAGATGAGGA[**C**]	Maxima SYBR Green/ROX qPCR master mix	60 °C	72 °C
			(R) TTTGCAGTTTATTAAGATGAGGA[**T**]	(Fermentas, Ontario, Canada)	40 seconds	1 minute
CCR5	rs553615728	(F) GTGGAGTAACGCACACTGCAA	(R) CCATTTCCTCATCTGTTAAATGAC[**G**]	FastStart Universal SYBR Green Master (Rox)	56 °C	72 °C
			(R) CCATTTCCTCATCTGTTAAATGAC[**A**]	(Roche, Basel, Switzerland)	1 minute	1 minute
	rs1799987	(R) TGGTGAGCATCTGTGTGG	(F) ACTTCACATTAACCCTGTG**T**	PowerUp™ SYBR® Green Master Mix	58 °C	72 °C
			(F) ACTTCACATTAACCCTGTG**C**	(Applied Biosystems, Massachusetts, USA)	30 seconds	30 seconds
	rs1015164	(R) GTGCCAAGCCTCCAGCTCTC	(F) CTGCCAGGGGACAATCACC**A**	PowerUp™ SYBR® Green Master Mix	62 °C	72 °C
			(F) CTGCCAGGGGACAATCACC**G**	(Applied Biosystems, Massachusetts, USA)	15 seconds	1 minute
	CCR5Δ32 (rs333)	(R) GATTCCCGAGTAGCAGATGACC	(F) TGCAGCTCTCATTTTCCATACAGT**C**	FastStart Universal SYBR Green Master (Rox)	58 °C	72 °C
			(F) TGCAGCTCTCATTTTCCATACAGT**A**	(Roche, Basel, Switzerland)	40 seconds	30 seconds
CXCR6	rs2234355	(R) GATATGACCAGCACCAGAGAG	(F) CAGAACAGACACCATGGCA[**A**]	Maxima SYBR Green/ROX qPCR master mix	60 °C	72 °C
			(F) CAGAACAGACACCATGGCA[**G**]	(Fermentas, Ontario, Canada)	40 seconds	1 minute
	rs2234358	(R) ACCAGCCAGAGTGTCTGATAA	(F) GCTGCTCTGGAATTTGCAAG[**G**]	Maxima SYBR Green/ROX qPCR master mix	60 °C	72 °C
			(F) GCTGCTCTGGAATTTGCAAG[**T**]	(Fermentas, Ontario, Canada)	40 seconds	1 minute

Variant base is bolded. Square brackets denote LNA™ modified 3' end nucleotides. (F) forward orientation, (R) reverse orientation. SNP: single nucleotide polymorphism.

2.7 Flow cytometry

2.7.1 Panel design

The expression of CCR5 or CXCR6 was analysed on total CD4+ ($CD3^+CD4^+$) and CD8+ ($CD3^+CD8^+$) T cells, NK cells ($CD3^-CD19^-CD14^-CD56^+CD16^+$ or $^-$), B cells (CD19+) and monocytes (CD14+), as well as on naïve ($CD45RO^-CCR7^+CD62L^+$), central memory ($CD45RO^+CCR7^+CD62L^+$), transitional memory ($CD45RO^+CCR7^-CD62L^+$), effector memory cells ($CD45RO^+CCR7^-CD62L^-$) and terminally differentiated ($CD45RO^-CCR7^-CD62L^-$) CD4+ and CD8+ T cells (166). Antibodies to HLA-DR were included to assess the extent of cell activation. Antibodies were titrated to determine the optimal volume of antibody to use in the panels that would allow for the best separation while giving the lowest background. Two panels were designed, one assessing CXCR6 and one assessing CCR5 expression, with 12 antibodies used in each panel. The monoclonal antibodies used are shown in Table 2.4.

Table 2.4 - Antibodies used in flow cytometry panels.

Antibody	Fluorochrome	Clone	Manufacturer
Anti-CD3	APC-H7	SK7	BD Biosciences
Anti-CD4	BV786	L200	BD Biosciences
Anti-CD8	PerCP	SK1	BD Biosciences
Anti-CD19	Alexa Fluor 700	HIB19	BD Biosciences
Anti-CD14	APC	M5E2	BD Biosciences
Anti-CD56	BV650	NCAM16.2	BD Biosciences
Anti-CD16	BV711	3G8	BD Biosciences
Anti-CD45RO	BV510	UCHL1	BD Biosciences
Anti-CCR7	FITC	150503	BD Biosciences
Anti-CD62L	PE-CF594	DREG-56	BD Biosciences
Anti-HLA-DR	PE-Cy5.5	TU36	Invitrogen
Anti-CXCR6	PE	K041E5	BioLegend
Anti-CCR5	PE	2D7	BD Biosciences

2.7.2 Whole blood staining

Whole blood was stained within 1 hour of sample collection. As multiple antibodies conjugated to Brilliant Violet (BV) fluorochromes were included in the panels, 50 µl of BD Brilliant Stain Buffer (BD Biosciences, San Jose, California, USA) was first added to 150 µl of whole blood, after which the antibodies were added. Samples were vortexed and incubated for 15 minutes at room temperature in the dark. To lyse red blood cells, 2 ml of FACS™ Lysing Solution (BD Biosciences, San Jose, California, USA), diluted 1:10 in distilled H_2O, was added and the samples were incubated for 10 minutes at room temperature in the dark. Samples were then centrifuged for 5 minutes at 2000 rpm, following which the supernatant was removed. Pellets were washed with 3 ml of FACSFlow™ (BD Biosciences, San Jose, California, USA) and resuspended in 250 µl of FACSFlow™ for acquisition on a four laser BD LSRFortessa™ X-20 Special Order Research

Product within 1 hour. Between 500 000 and 1 million total events were acquired per sample in order to ensure sufficient cells were available for analysis.

2.7.3 Quantitation

PE-labeled antibodies to CCR5 and CXCR6, together with the commercially available BD QuantiBRITE™ Beads PE Fluorescence Quantitation Kit system (BD Biosciences, San Jose, California, USA), were used to quantitate the number of molecules of CCR5/CXCR6 per cell. Every QuantiBRITE tube contains a pellet of lyophilised beads with four different levels of PE covalently attached. A linear regression equation can be calculated using the geometric means and the PE molecules/bead from the QuantiBRITE™ tube. Since the QuantiBRITE™ tube is acquired at the same instrument settings as the samples, this equation can then be used to convert the CCR5/CXCR6 geometric means on the cell subsets of interest into the number of PE molecules bound per cell. QuantiBRITE™ beads with 474, 5 359, 23 843 and 62 336 PE molecules/bead, for the low, med-low, med-high and high beads respectively, were used for all experiments.

2.7.4 Flow cytometry quality control and standardization

2.7.4.1 CS&T Beads

Before each experiment, BD™ CS&T Beads (BD Biosciences, San Jose, California, USA) were run for quality control of the instrument's optics, electronics, and fluidics. The beads assess the detector performance and measure the sensitivity of each fluorescence detector, which is a measure of the cytometer's ability to resolve cells that are dimly stained. The software calculates the bright bead median, bright beads percentage of robust coefficient of variation (rCV) and instrument sensitivity for forward scatter (FSC), side scatter (SSC), and each fluorescence parameter, and compares them to expected values for the bead lot. The rCV measures cytometer alignment.

2.7.4.2 Rainbow beads

When setting up the flow cytometry experiments, the sensitivity of the detectors was optimised for instrument performance. However, because of daily variations as well as cytometer maintenance and laser realignment, voltages were adjusted before each experiment to ensure reproducibility. Mid-range Rainbow Fluorescent Particles (BD Biosciences, San Jose, California, USA) are

uniform size particles that are dyed with a mix of fluorophores which can be excited from 365 to 650 nm. They produce a single peak, with tight CVs, in each detection channel. When acquiring the rainbow beads for the first time, a set of baseline target mean fluorescence intensity (MFI) values was generated. At the start of each subsequent day of acquisition, rainbow beads were acquired and the voltages were adjusted until the MFI of the rainbow beads was within 10 percent of the target MFI for each detector. The baseline target MFIs for all detectors in the CCR5 and CXCR6 panel are shown in Figure 2.3.

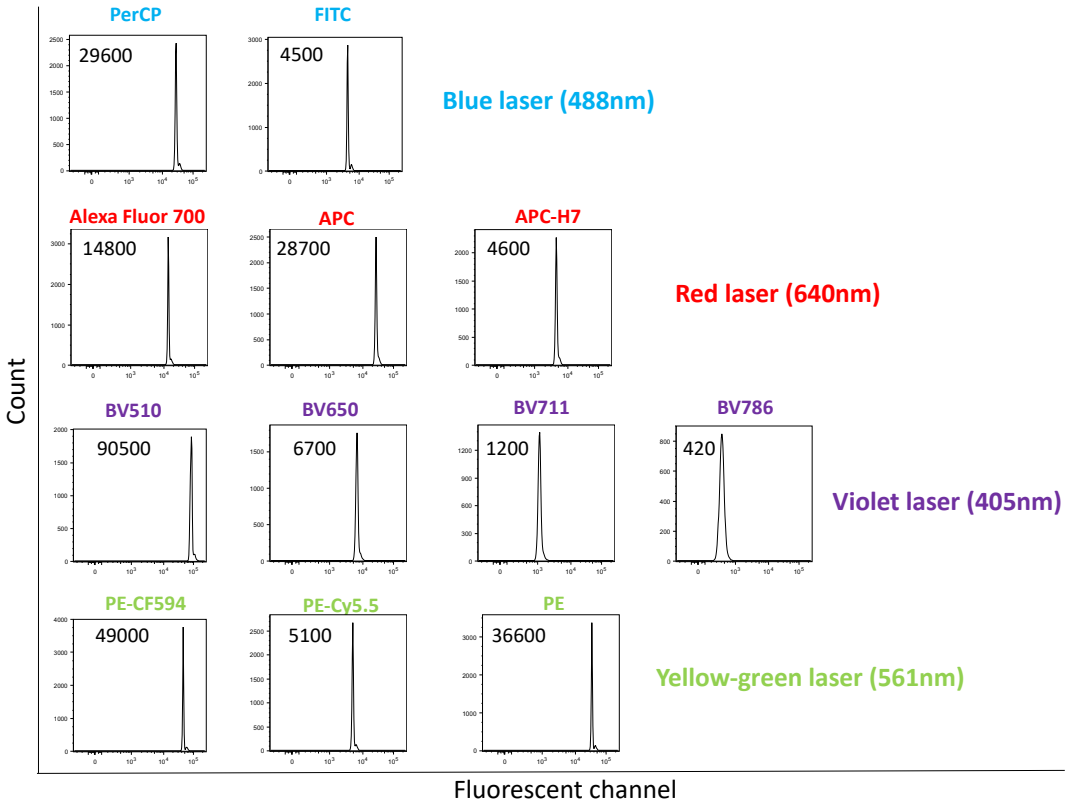


Figure 2.3 - Histograms depicting a signal detection from rainbow beads in all channels used in the CCR5 and CXCR6 panels. The baseline target values for the respective channels are shown. The excitation laser wavelength for the respective fluorochromes is shown.

2.7.4.3 Compensation controls

The emission spectra of most fluorochromes is very broad. Therefore, the fluorescent signal from one fluorochrome can be detected in detectors other than the detector that is supposed to measure it (primary detector). This is known as spillover or spectral overlap. Compensation corrects for spectral overlap by mathematically removing the fluorescent signal from all detectors except the primary detector. Compensation is measured as a percentage.

Compensation controls are single-stained samples for each antibody in the panel. Compensation is specific for the fluorochrome and not the cell type, so beads can be used for compensation. The BD™ CompBeads set (BD Biosciences, San Jose, California, USA) was used to perform compensation before every experiment. This set has one tube containing BD™ CompBeads Anti-Mouse Ig, κ particles which will bind any mouse κ light chain immunoglobulin (positive) and

another tube containing the BD™ CompBeads Negative Control which does not have any capacity to bind. These compensation beads provide distinct negative (background fluorescence) and positive stained populations which can be used to calculate compensation. Histograms depicting unstained and stained compensation beads for all fluorochromes used in the CCR5 and CXCR6 panels are shown in Figure 2.4.

One drop each of negative and positive compensation beads was added to 100 µl of FACSFlow™ in each compensation tube together with the appropriate antibody. Each tube was vortexed and incubated for 15 minutes at room temperature in the dark, after which 2 ml of FACSFlow™ was added. The tube was then centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded and another 2 ml of FACSFlow™ was added before repeating centrifugation. The supernatant was poured off and 250 µl of FACSFlow™ was added.

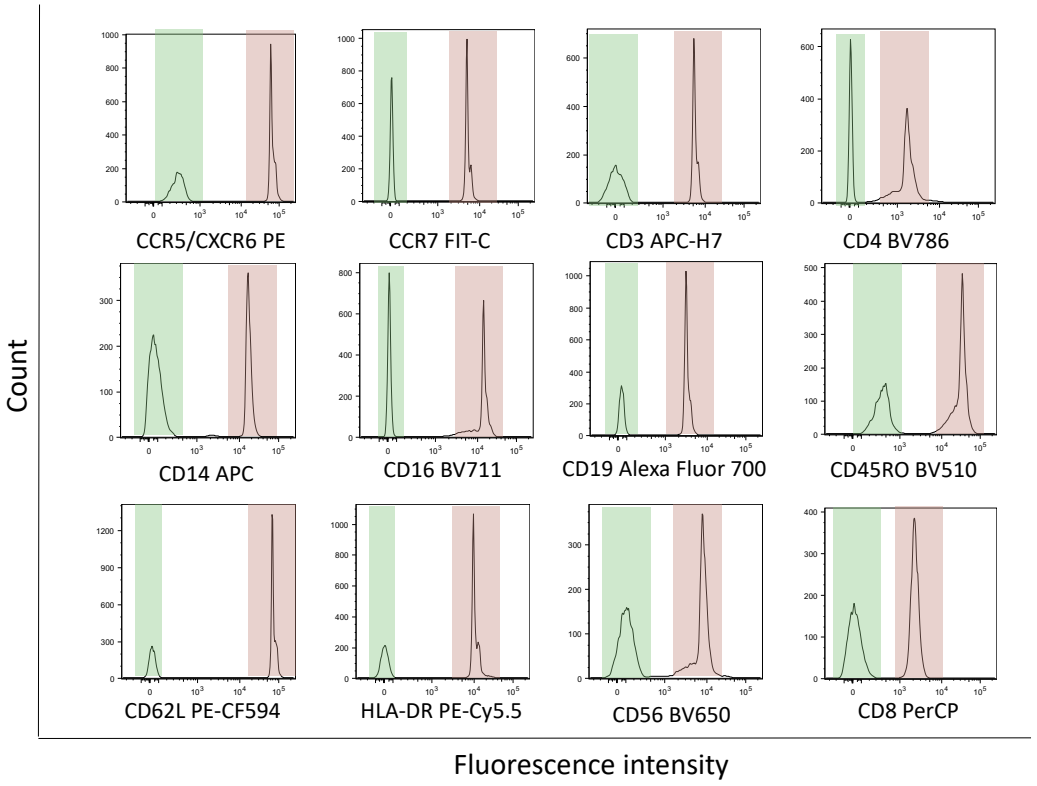


Figure 2.4 – Representative histograms of unstained (green) and stained (red) compensation beads for all fluorochromes used in the CCR5 and CXCR6 panels.

2.7.5 Gating controls

2.7.5.1 Fluorescence Minus One (FMO) controls

Fluorescence minus one (FMO) controls are gating controls. An FMO control contains cells stained with all fluorochromes in the panel except one. This ensures that the spread of fluorochromes into the unlabelled channel is identified, and therefore assists in determining the correct position for the gate. FMO controls were run for both the CXCR6 and the CCR5 panel and assisted with gating of cell populations.

2.7.6 Gating strategies

Flow cytometry data were analysed using FlowJo 9.9.6 (Tree Star, San Carlos, California, USA). The gating strategies quantifying CCR5 and CXCR6 expression on CD4+ and CD8+ T cells and subsets (naïve, central memory, transitional memory, effector memory and terminally differentiated), B cells, monocytes and subsets ($CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$, $CD14^{+}CD16^{bright}$) and NK cells and subsets ($CD56^{bright}$, $CD56^{dim}$, $CD56^{+}CD16^{+}$, $CD56^{bright}CD16^{+}$, $CD56^{dim}CD16^{+}$, $CD56^{bright}CD16$, $CD56^{dim}CD16^{-}$) are shown in Figures 2.5 – 2.9, respectively.

2.7.6.1 CD4+ T cells

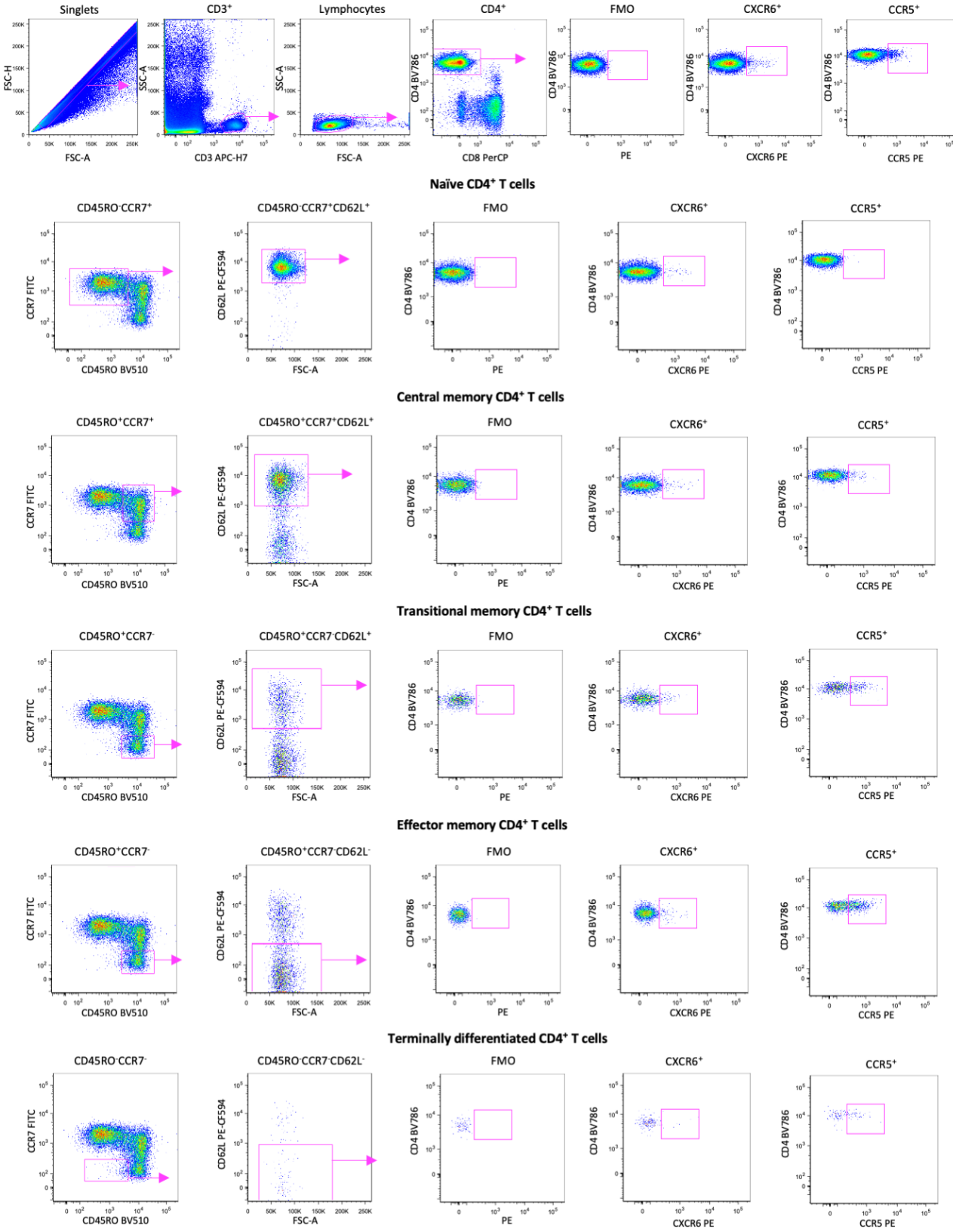


Figure 2.5 - Representative gating strategy for naïve and memory CD4+ T cell. Singlets were gated using FSC-A vs FSC-H followed by gating of CD3+ T cells. CD3+ T cells were further gated using low SSC-A vs low FSC-A. CD4+ cells were divided into four populations based on their differential CCR7 and CD45RO expression. These populations were subsequently analysed for their CD62L expression to define naïve ($CD45RO^{-}CCR7^{+}CD62L^{+}$), central memory ($CD45RO^{+}CCR7^{+}CD62L^{+}$), transitional memory ($CD45RO^{+}CCR7^{-}CD62L^{+}$), effector memory ($CD45RO^{+}CCR7^{-}CD62L^{-}$) and terminally differentiated ($CD45RO^{-}CCR7^{-}CD62L^{-}$) CD4+ T cells which were then examined for CXCR6 and CCR5 expression. The FMO controls are shown.

2.7.6.2 CD8+ T cells

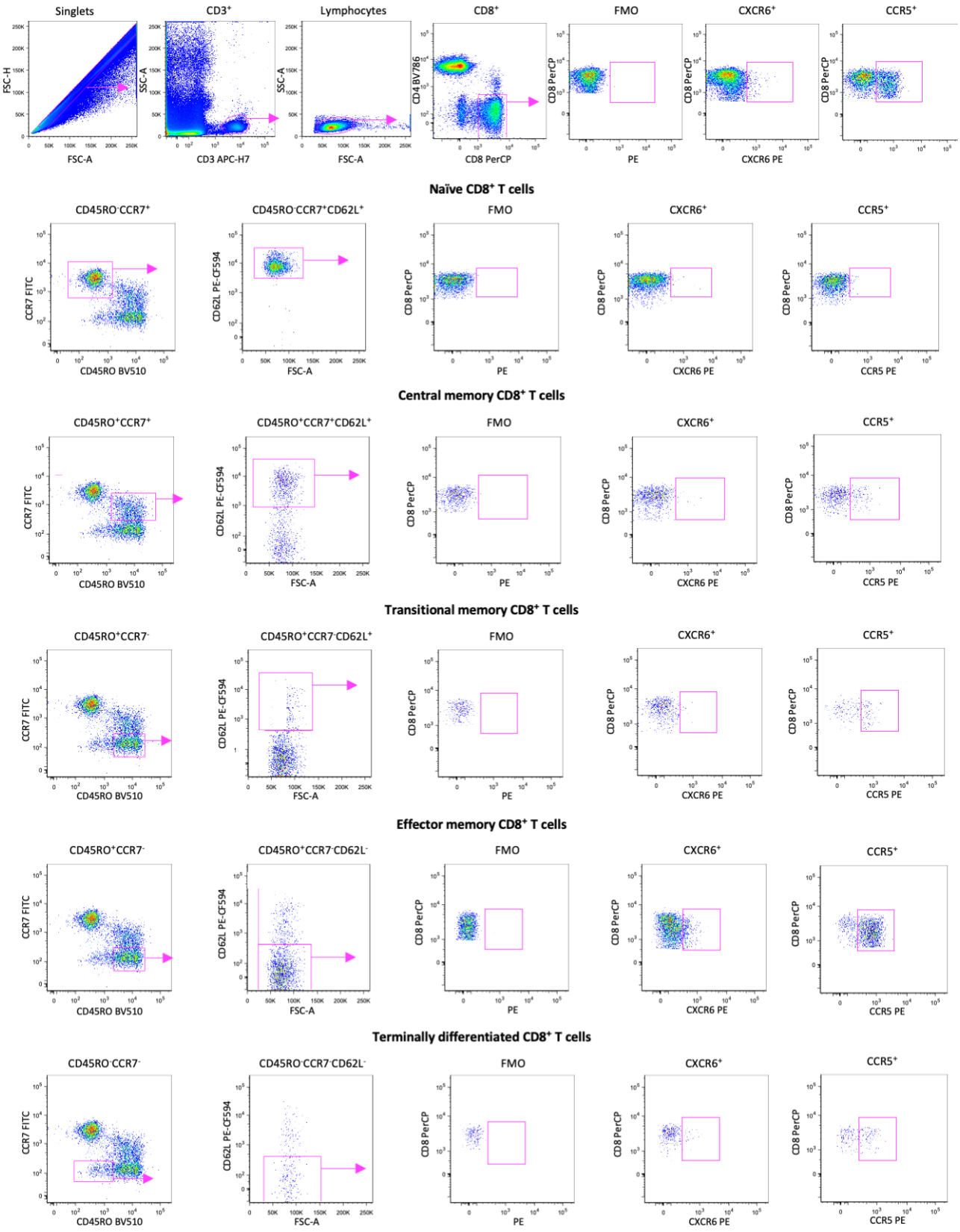


Figure 2.6 - Representative gating strategy for naïve and memory CD8+ T cell subsets. Singlets were gated using FSC-A vs FSC-H followed by gating of $CD3^+$ T cells. CD3+ T cells were further gated using low SSC-A vs low FSC-A. CD8+ cells were divided into four populations based on their differential CCR7 and CD45RO expression. These populations were subsequently analysed for their CD62L expression to define naïve ($CD45RO^-CCR7^+CD62L^+$), central memory ($CD45RO^+CCR7^+CD62L^+$), transitional memory ($CD45RO^+CCR7^-CD62L^+$), effector memory ($CD45RO^+CCR7^-CD62L^-$) and terminally differentiated ($CD45RO^-CCR7^-CD62L^-$) CD8+ T cells which were then examined for CXCR6 and CCR5 expression. The FMO controls are shown.

2.7.6.3 B cells

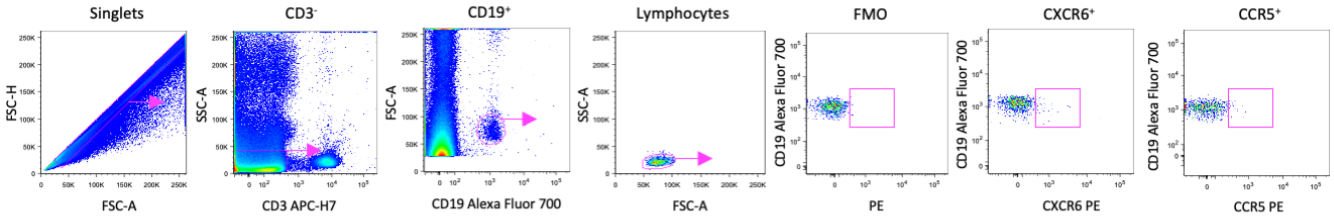


Figure 2.7 - Representative gating strategy for B cells. Singlets were gated using FSC-A vs FSC-H. B cells were identified by CD19 gating of CD3- cells, followed by gating on low SSC-A vs low FSC-A. CD19+ B cells were then examined for CXCR6 and CCR5 expression. The FMO control is shown.

2.7.6.4 Monocytes

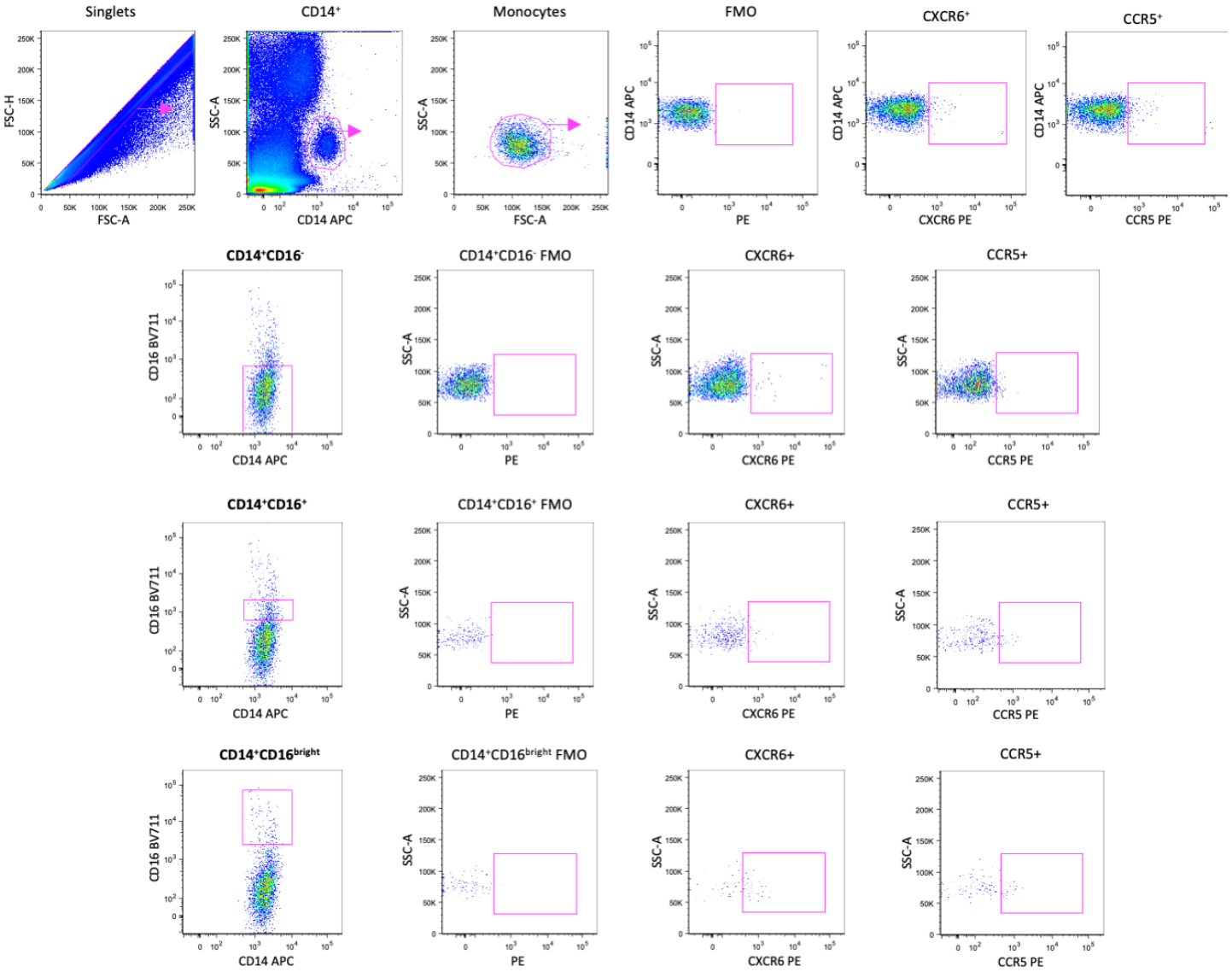


Figure 2.8 - Representative gating strategy for monocyte subsets. Singlets were gated using FSC-A vs FSC-H. CD14+ monocytes were divided into three populations based on their CD14 and CD16 expression, which were then examined for CXCR6 and CCR5 expression. FMO controls are shown.

2.7.6.5 NK cells

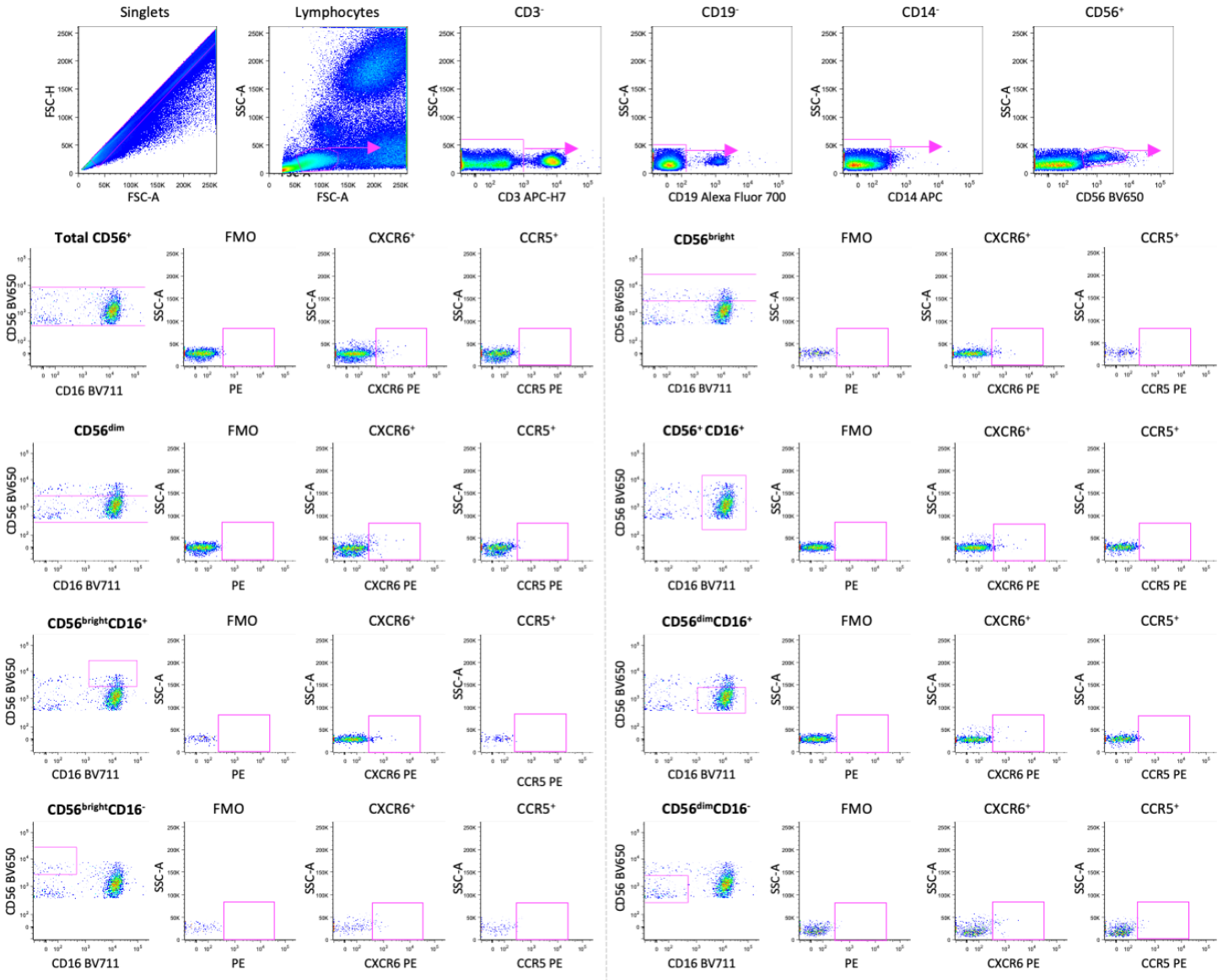


Figure 2.9 - Representative gating strategy for natural killer cell subsets. Singlets were gated using FSC-A vs FSC-H. Lymphocytes were gated according to their SSC-A and FSC-A properties. CD3-, CD19-, CD14- and CD56+ cells were divided into seven CD56+ NK cell subsets, based on their differential expression of CD56 and CD16, which were then examined for CXCR6 and CCR5 expression. FMO controls are shown.

2.8 Assessing the effect of *CCR5* and *CXCR6* SNPs on expression levels

The *CCR5* -4223 C>T (rs553615728), -2459 G>A (rs1799987), rs1015164 G>A, +2919 T>G (rs746492) and *CXCR6* rs2234355 G>A and rs2234358 G>T polymorphisms were analysed with regards to expression levels, as measured by flow cytometry, by stratifying individuals' CCR5 and CXCR6 expression levels into different *CCR5* and *CXCR6* genotype groupings to investigate the potential association of variants and background expression.

2.9 Flow cytometry data analysis

Flow cytometry data was assessed and CCR5 or CXCR6 expression analysed both as a measure of frequency of expression (i.e., percentage expression) and as a measure of density (i.e., number of molecules/cell – refer to section 2.7.3). Proportions of T cell subsets were determined by calculating the percentage of CD4+ and CD8+ T cell subsets of an individual's CD3+ cells. Ratios of expression were calculated by dividing an individual's CCR5 expression by CXCR6 expression.

2.10 Statistical analyses

The genotypic data generated for polymorphic loci were tested for linkage disequilibrium as well as deviation from Hardy-Weinberg equilibrium using Haploview version 4.2 software (167). VassarStats: website for statistical computation (http://vassarstats.net/odds2x2.html) and Statistica™ software (TIBCO software, Palo Alto, California, USA) was used to compare SNP and haplotype frequencies between respective groups and to calculate statistical significances and exact 95% confidence intervals (CI) of odds ratios (OR). Corrections for multiple comparisons were applied using the False Discovery Rate (FDR) and Bonferonni-Dunn tests. Analyses were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, California, USA www.graphpad.com). The frequency of each haplotype was calculated by counting the number of haplotypic alleles and dividing by the total number of alleles. Visual analysis was used for determining haplotypes across gene regions. Non-parametric Mann-Whitney U tests were used to compare flow cytometry data between black and white South Africans and between males and females, and Spearman's correlation coefficient was used to describe associations between CCR5 and CXCR6 expression and participant age and cellular activation (HLA-DR expression), and to correlate CCR5 expression with CXCR6 expression. For all analyses, two-sided tests were used and statistical significance for all analyses was set at $p<0.05$.

CHAPTER 3

3. *Cis*-regulatory genetic variants in the *CCR5* gene and natural HIV-1 control in black South Africans

3.1 Introduction

Unique individuals who are able to either naturally suppress HIV-1 viral load (VL) or maintain healthy CD4+ T cell counts with higher viral loads, and who exhibit slow progression of the disease without antiretroviral therapy (ART), are broadly termed HIV-1 controllers. Within this group there are rare individuals termed elite controllers (ECs) who suppress VL to less than 50 RNA copies/ml plasma, viraemic controllers (VCs) who tend to have low viral load set-points (generally <2000 RNA copies/ml) with sustained high CD4+ T cell counts, and high viral load long-term non-progressors (HVL LTNPs) who maintain high CD4+ T-cell counts for prolonged periods without ART despite high viral loads (generally >10 000 RNA copies/ml), a similar phenotype to SIV-infected sooty mangabeys (67). A number of factors, including viral, immunological and environmental, have been proposed to influence the ability of an individual to naturally control HIV-1 (168), however it is likely that different mechanisms may be responsible for the different modes of control.

The chemokine receptor CCR5, together with the CD4 receptor, is responsible for allowing HIV-1 entry into the target cell and is well studied with regards to HIV-1 disease. Lower CCR5 expression levels have been associated with slower HIV-1 disease progression (96). The differential expression of CCR5 may be explained by polymorphisms in the *cis*-regulatory regions of *CCR5* (169). Although the 5'UTR has been extensively studied, surprisingly, little is known about the genetic variation in the *CCR5* 3'UTR, a region increasingly shown to be crucial for mRNA regulation.

One of the most studied genetic variations in *CCR5* is the Δ32 mutation (*CCR5Δ32*), a 32 base pair deletion that results in non-functional CCR5 receptors. This variant is predominantly found in European populations and is virtually absent in African, East Asian, and American Indian populations (108). The rs553615728 C>T single nucleotide polymorphism (SNP) has been shown to disrupt a cytidine phosphate guanidine (CpG) dinucleotide in the *cis*-region of *CCR5,* namely

CpG-41, a binding site where DNA methylation occurs, and is uniquely found in individuals from southern Africa (115). Additionally, there are nine previously described *CCR5* 5'UTR haplotypes (109–111) that have been studied with respect to HIV-1 disease progression (HHA, HHB, HHC, HHD, HHE, HHF*1, HHF*2, HHG*1, HHG*2). These haplotypes are defined by seven *CCR5* 5'UTR SNPs and the presence/absence of *CCR5Δ32* and a SNP in the coding region of chemokine receptor *CCR2* (*CCR2-V64I*) (110,111).

This study was therefore conducted to explore the genetic variation in the *cis*-regulatory regions of *CCR5* and to explore the role of these variants on HIV-1 control in a population of black South African controllers and progressors living with HIV-1. This information will help to formulate a protective and deleterious *CCR5* genetic signature for black South African individuals living with HIV-1 and will be useful in informing cure strategies.

3.2 Materials and Methods

3.2.1 Sample population

Refer to Chapter 2, Materials and Methods section 2.1.1. Briefly, black South African ART-naïve elite controllers (n=23), viraemic controllers (n=37), high viral load long-term non-progressors (n=11) and progressors (who required initiation of ART upon enrolment; n=74) living with HIV-1 were recruited for this study.

3.2.2 Standard polymerase chain reaction (PCR) amplification and sequencing of *CCR5* 5'UTR and 3'UTR

Refer to Chapter 2, Materials and Methods section 2.2 and 2.3.

3.2.3 Sequence analysis

Refer to Chapter 2, Materials and Methods section 2.4.

3.2.4 CCR5 haplotype assignment

Refer to Chapter 2, Materials and Methods section 2.5.

3.2.5 Single nucleotide polymorphism genotyping

Refer to Chapter 2, Materials and Methods section 2.6.

3.2.6 Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium

The genotypic data generated for polymorphic loci were tested for linkage disequilibrium as well as deviation from Hardy-Weinberg equilibrium using the Haploview version 4.2 software (167).

3.2.7 Analysis and statistics

Refer to Chapter 2, Materials and Methods section 2.10.

3.3 Results

3.3.1 *CCR5* 5'UTR: variability and HIV-1 control

We amplified and sequenced the *CCR5* promoter region and individuals were genotyped for the *CCR5*Δ32 deletion and the *CCR2-V64I* polymorphism. Previously defined haplotypes (111,116) were assigned to the 71 HIV-1 controllers and 74 HIV-1 progressors. The allelic representation and percentage of these haplotypes in controllers, controller subgroups and progressors is shown in Figure 3.1. Interestingly, one individual in our study, a viraemic controller, was heterozygous for the *CCR5Δ32* deletion (haplotype HHG*2).

With regards to CCR5 promoter haplotypes, the most prominent relationship was seen with respect to the HHE haplotype. All controller subgroups showed low levels of HHE representation compared to progressors (21.6%; Figure 3.1), however this was only significant in the VCs (6.8%; p=0.006; OR=3.81; CI =1.42-10.23) and total controllers (9.2%; p=0.003; OR=2.74; CI=1.37-5.47). The combination of select CCR5 haplotypes (i.e., the genotypes) and the presence or absence of a particular allele (haplotype) collectively influence CCR5 expression. HHA/HHC was significantly overrepresented in the total group of controllers compared to the progressors (p=0.028; OR=0.19; CI=0.04-0.92) and this was more significant in the VCs compared to the progressors (p=0.016; OR=0.14; CI=0.03-0.75). The large confidence intervals of these findings suggest that further study of HHA/HHC in larger cohorts would be helpful to determine true significance. The HHD/HHG*1 genotype was also overrepresented in the total group of controllers and VCs compared to the progressors (p=0.054 and p=0.040, respectively). More significantly, exclusion of ECs, i.e., grouping the VCs + HVL LTNPs (n=48), revealed the HHD/HHG*1 genotype to be overrepresented in this group compared to progressors (8.3% vs 0%; p=0.020). The

frequencies of all *CCR5* haplotype genotypes detected in the controllers, controller subgroups and progressors are shown in Table 3.1.

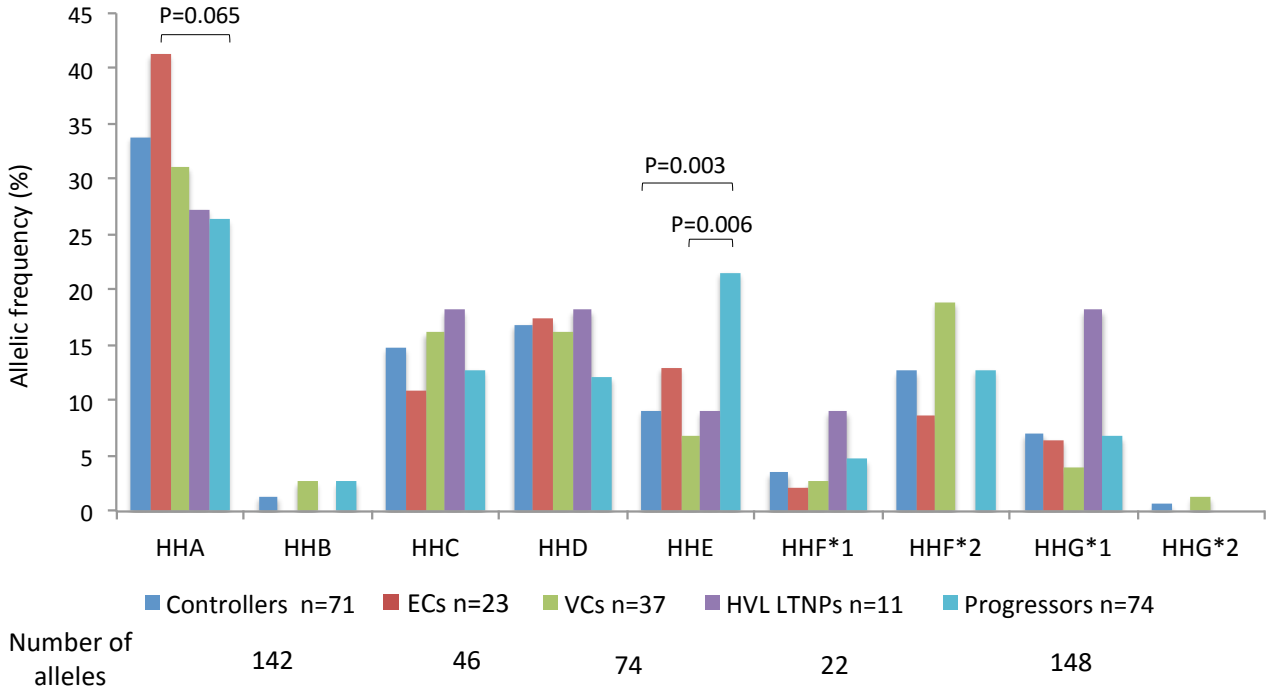


Figure 3.1 – Bar graph showing the allelic frequencies (%) of previously defined CCR5 promoter haplotypes in black South Africans living with HIV-1.

Table 3.1 - Genotypic frequencies of CCR5 promoter haplotypes in black South African controllers, controller subgroups and progressors.

CCR5 haplotype genotype	Controllers (n=71)	ECs (n=23)	VCs (n=37)	HVL LTNPs (n=11)	Progressors (n=74)
HHA/HHA	9	4	4	1	7
HHA/HHB	0	0	0	0	0
HHA/HHC ◆◆	9	2	6	1	2
HHA/HHD	8	3	4	1	5
HHA/HHE	4	2	2	0	9
HHA/HHF*1	2	0	1	1	2
HHA/HHF*2	4	2	2	0	4
HHA/HHG*1	2	1	0	1	3
HHB/HHB	0	0	0	0	0
HHB/HHC	1	0	1	0	2
HHB/HHD	0	0	0	0	0
HHB/HHE	0	0	0	0	1
HHB/HHF*1	0	0	0	0	0
HHB/HHF*2	1	0	1	0	1
HHB/HHG*1	0	0	0	0	0
HHC/HHC	2	1	1	0	1
HHC/HHD	3	1	1	1	1
HHC/HHE ◆	1	0	0	1	7
HHC/HHF*1	1	0	1	0	2
HHC/HHF*2	1	0	1	0	3
HHC/HHG*1	1	0	0	1	0
HHD/HHD	2	1	1	0	0
HHD/HHE ◆	2	1	0	1	8
HHD/HHF*1	0	0	0	0	1
HHD/HHF*2	2	0	2	0	3
HHD/HHG*1 ◆	3	0	2	1	0
HHD/HHG*2	1	0	1	0	0
HHE/HHE	0	0	0	0	1
HHE/HHF*1	1	1	0	0	0
HHE/HHF*2	3	0	3	0	2
HHE/HHG*1	0	0	0	0	3
HHF*1/HHF*1	0	0	0	0	0
HHF*1/HHG*1	1	0	0	1	2
HHF*2/HHF*2	2	0	2	0	3
HHF*2/HHG*1	3	2	1	0	0
HHG*1/HHG*1	0	0	0	0	1

◆ Comparison trending to significance ◆◆ Significant comparison

Two 5'UTR SNPs, common to the HHE, HHF and HHG haplotypes (-2459 A and -2135 C alleles), were in complete linkage disequilibrium in our study population (LD; r^2=1). We hence analysed the effect of this 2-SNP haplotype (termed 5'UTR-2SNP-hap) alone. Table 3.2 shows results of the comparison of representation of 5'UTR-2SNP-hap between controller and progressor groups and Figure 3.2 depicts its genotypic frequency in the respective groups. The minor allele of 5'UTR-2SNP-hap was significantly underrepresented in the total controller group compared to progressors (p=0.031; OR=1.72; CI=1.07-2.77). Heterozygosity for 5'UTR-2SNP-hap was significantly less prevalent in ECs compared to progressors (p=0.016; OR=3.67: CI=1.28-10.47), VCs compared to progressors (p=0.012; OR=3.38; CI=1.38-8.32) and most significantly in the total controller group compared to progressors (p=0.003; OR=3.08; CI=1.46-6.49, $p_{bonferroni}$=0.048). Interestingly, heterozygosity for 5'UTR-2SNP-hap was very similar between HVL LTNPs and progressors (55% vs 59%). Comparison of the 5'UTR-2SNP-hap in the dominant mode (i.e., wild type (WT)/mutant (Mt) + Mt/Mt) revealed similar results (Table 3.2). The genotypic frequencies of all identified 5'UTR SNPs in the respective groups are shown in Table 3.3.

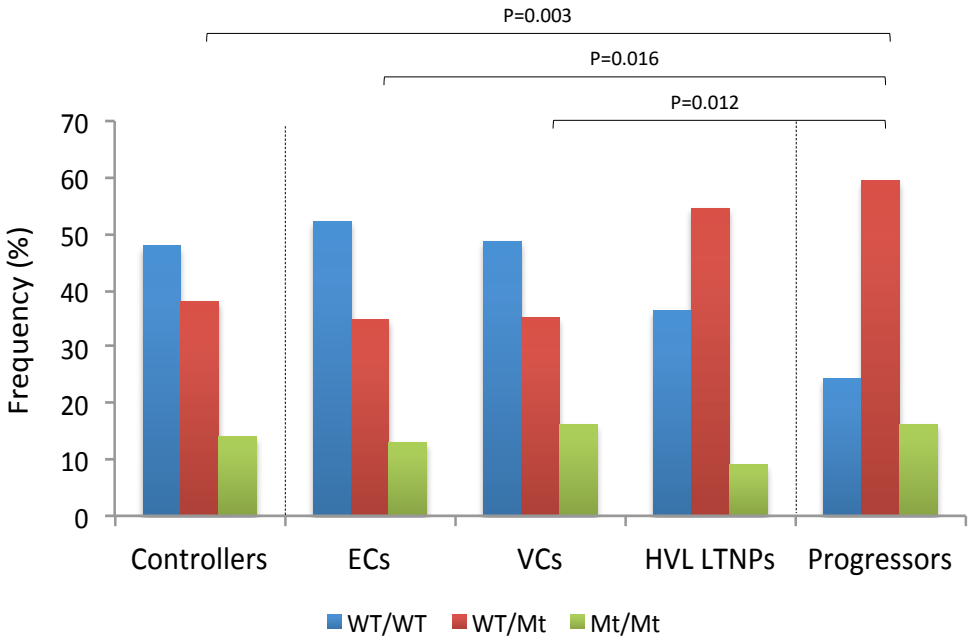


Figure 3.2 - Bar graph showing the genotypic frequency of 5'UTR-2SNP-hap (-2459 G>A and -2135 T>C) in black South African controllers, controller subgroups (ECs, VCs and HVL LTNPs) and progressors. ECs: elite controllers, VCs: viraemic controllers, HVL LTNPs: high viral load long-term non-progressors, WT: wild type, Mt: mutant.

Table 3.2 - Comparison of the genotypic and allelic representation of CCR5 5'UTR-2SNP-hap (-2459 G>A and -2135 C>T) and CCR5 3'UTR +2919 T>G SNP in controllers, controller subgroups and progressors.

	Controllers vs. Progressors			ECs vs. Progressors			VCs vs. Progressors			HVL LTNPs vs. Progressors		
Polymorphism	**OR**	**CI**	***P***	**OR**	**CI**	***P***	**OR**	**CI**	***P***	**OR**	**CI**	***P***
5'UTR-2SNP-hap												
5'UTR-2SNP-hap allelic	1.72	1.07-2.77	0.03	1.94	0.96-3.94	0.087	1.67	0.93-2.98	0.11	1.49	0.59-3.76	0.49
5'UTR-2SNP-hap heterozygosity (WT/Mt)	3.08	1.46-6.49	0.003 (0.048*)	3.67	1.28-10.47	0.017	3.38	1.38-8.32	0.012	1.63	0.41-6.47	0.71
5'UTR-2SNP-hap dominant mode	2.86	1.41-5.79	0.003 (0.048*)	3.39	1.28-9.00	0.019	2.95	1.28-6.79	0.017	1.78	0.47-6.78	0.46
3'UTR +2919 SNP												
+2919T>G SNP allelic	1.9	1.18-3.09	0.01	1.89	0.93-3.83	0.08	1.83	1.02-3.31	0.06	2.21	0.82-5.95	0.17
+2919T>G SNP heterozygosity (WT/Mt)	3.33	1.59-7.00	0.002 (0.032*)	2.75	1.00-7.60	0.06	3.7	1.5-8.92	0.004	3.75	0.95-14.88	0.07
+2919T>G SNP dominant mode	3.2	1.58-6.48	0.001 (0.016*)	2.85	1.08-7.56	0.04	3.28	1.43-7.57	0.006	3.73	1.02-13.70	0.07

Asterisks indicate p value after Bonferroni correction for multiple comparisons. Other comparisons did not maintain significance after Bonferroni correction. Shaded blocks indicate significant comparisons. ECs: elite controllers, VCs: viraemic controllers, HVL LTNPs: high viral load long-term non-progressors, OR: odds ratio, CI: confidence interval, WT: wild type, Mt: mutant.

Table 3.3 - Genotypic frequencies of SNPs found in the *CCR5* 5'UTR and the *CCR5* 3'UTR in black South African controllers, controller subgroups and progressors.

	CCR5 5'UTR POLYMORPHISMS							CCR5 3'UTR POLYMORPHISMS										
	-2733	**-2554**	**-2459**	**-2135**	**-2132**	**-2086**	**-1838**	**+1752**	**+1843**	**+1846**	**+2066**	**+2077**	**+2225**	**+2381**	**+2458**	**+2772**	**+2838**	**+2919**
Controllers (n=71)																		
WT/WT	60	32	34	34	49	52	49	29	49	49	67	53 *	56 *	68	59	66	62	36
WT/Mt	11	31	27	27	20	17	20	33	19	19	4	14 *	12 *	3	10	5	8	27
Mt/Mt	0	8	10	10	2	2	2	9	3	3	0	2 *	2 *	0	2	0	1	8
ECs (n=23)																		
WT/WT	20	11	12	12	15	18	18	6	16	16	22	20	16 *	23	20	21	19	11
WT/Mt	3	9	8	8	8	4	5	13	5	5	1	2	4 *	0	2	2	3	10
Mt/Mt	0	3	3	3	0	1	0	4	2	2	0	1	2 *	0	1	0	1	2
VCs (n=37)																		
WT/WT	33	17	18	18	27	27	23	17	27	27	35	26 *	31	34	30	35	33	19
WT/Mt	4	16	13	13	8	9	12	16	9	9	2	8 *	6	3	6	2	4	13
Mt/Mt	0	4	6	6	2	1	2	4	1	1	0	1 *	0	0	1	0	0	5
HVL LTNPs (n=11)																		
WT/WT	7	3	4	4	7	7	8	6	6	6	10	7	9	11	9	10	10	6
WT/Mt	4	4	6	6	4	4	3	4	5	5	1	4	2	0	2	1	1	4
Mt/Mt	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Progressors (n=74)																		
WT/WT	64	36	18	18	55	56	50	32	51	51	70	59	57	67	67	61	63	18
WT/Mt	10	34	44	44	19	17	21	37	22	22	3	15	16	7	7	13	10	45
Mt/Mt	0	4	12	12	0	1	3	5	1	1	0	0	1	0	0	0	1	11

Asterisks indicate incomplete data. For the +2077 SNP, genotypes for two VCs are missing (n=69) and for the +2225 SNP, one EC genotype is missing (n=70). ECs: elite controllers, VCs: viraemic controllers, HVL LTNPs: high viral load long-term non-progressors, WT: wild type, Mt: mutant.

The *CCR5* -4223 C>T SNP had previously been genotyped for a subset of these individuals (52 controllers and 66 progressors) (115). We developed an allelic discrimination assay and genotyped the remaining 19 controllers and 8 progressors. Although there is very strong LD between the -4223 C>T SNP and the HHA haplotype in our study, one individual out of the twelve possessing the -4223 T allele in the complete cohort (controllers and progressors) did not possess an HHA haplotype (a progressor). Among controllers, the -4223 C>T SNP was exclusively found in the non-EC controllers. Furthermore, we noted that three out of five progressors that harboured the -4223 C>T SNP also possessed the HHE haplotype. Viraemic controllers had significantly higher representation of the -4223 C>T SNP in the absence of HHE compared to progressors (p=0.04; OR=0.18; CI=0.03-0.97), and when we compared the non-EC controllers to progressors in the absence of HHE, this relationship was strengthened (p=0.03; OR=0.16; CI=0.03-0.82).

3.3.2 *CCR5* 3'UTR: variability and HIV-1 control

Sequencing the *CCR5* 3'UTR and analyzing the resulting chromatograms revealed eleven previously reported variants at positions +1752 (rs41495153), +1843 (rs41418945), +1846 (rs41466044), +2066 (170), +2077 (rs1800874), +2225 (rs41535253), +2381 (rs550958125), +2458 (rs3188094), +2772 (170), +2838 (rs41512547) and +2919 (rs746492). The genotypic frequencies of all identified 3'UTR variants in the respective groups are shown in Table 3.3.

A *CCR5* 3'UTR small insertion or deletion of bases (indel; G insertion) at position +2772 was identified in HIV-1 uninfected black and Caucasian South African individuals (170). In our study, this indel was found to be underrepresented in the total controllers compared to progressors (p=0.08; OR=2.8; CI=0.95-8.36). Additionally, the 3'UTR +2919 T>G SNP was differentially represented between controllers and progressors. Table 3.2 shows results of the comparison of representation of the 3'UTR +2919 T>G SNP between controller and progressor groups. The minor allele (G) showed trends of lower representation in both ECs and VCs compared to progressors and was significantly underrepresented in the total controllers compared to progressors (p=0.01; OR=1.90; CI=1.18-3.09). Comparison of +2919 T>G genotypes showed heterozygosity (TG) to be underrepresented in all controller groups compared to progressors, and was significant in the VCs (p=0.004; OR=3.65; CI=1.50-8.92) and the total group comparison (p=0.002; OR=3.33; CI=1.59-6.99, $p_{bonferroni}$=0.032). Comparison of the +2919 T>G SNP in the dominant mode (i.e., TG+GG) revealed the most significant results however, with lower representation in all controller subgroups

compared to progressors, that were significant in ECs (p=0.04; OR=2.85; CI=1.08-7.56), VCs (p=0.006; OR=3.28; CI=1.42-7.57), but most significant in the total controller group comparison with 49.3% representation in the controllers compared to 75.7% in progressors (p=0.001; OR=3.2; CI=1.58-6.48, $p_{bonferroni}$=0.016).

Visual examination of the data revealed obvious linkage between select SNPs in the *CCR5* 3'UTR and were thus analysed as haplotypes. The linkage disequilibrium (LD) plot showing linkage between the 3'UTR SNPs is shown in Figure 3.3. The polymorphisms making up each haplotype and the allelic frequency of these haplotypes in controllers, controller subgroups and progressors is shown in Figure 3.4 A and B, respectively. Linkage patterns between the SNPs in the *CCR5* 3'UTR were complex and interesting. The +1843 G>A and +1846 G>A SNPs were in complete LD (r^2=1) and had directional LD with the +1752 G>A SNP (r^2=0.4) i.e., they always occurred with the upstream +1752G>A SNP (3'UTR-hap1), however the +1752 G>A SNP was found in the absence of the +1843 G>A and +1846 G>A haplotype. The +2225 T>C SNP also had directional LD with 3'UTR-hap1 (r^2=0.66; 3'UTR Hap1a) and the +2838 C>G SNP had directional LD with 3'UTR-hap1a. The +2458 A>C SNP had directional linkage with the +2077 G>T SNP (r^2=0.54; 3'UTR-hap2). Except for 7 individuals (6 controllers and 1 progressor), the +2077 G>T SNP never occurred with 3'UTR-hap1a (43/50). When occurring together, the +2066 G>A and +2077 G>T SNPs were in complete directional LD with the +2772 indel (r^2=1; 3'UTR-hap3). The +2381 A>G SNP had directional linkage with the +1752 SNP G>A (r^2=1; 3'UTR-hap4).

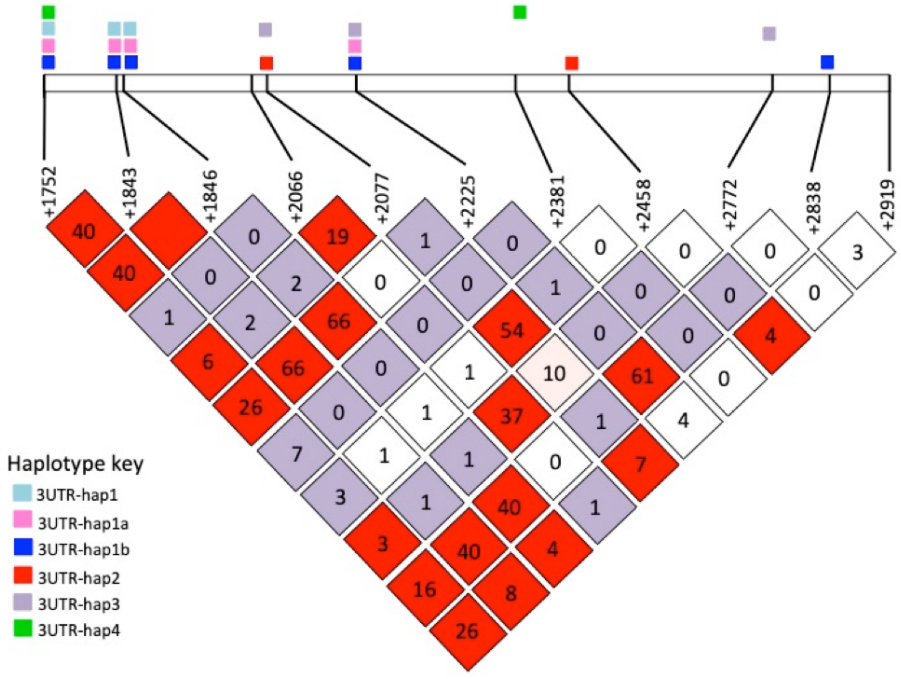


Figure 3.3 - Linkage disequilibrium (LD) plot depicting the linkage between CCR5 3'UTR variants. The numbers in the triangles indicate the r^2 values, with 0 indicating no linkage and blank red triangles (100) indicating complete linkage between the respective variants. Polymorphisms making up the various 3'UTR haplotypes are marked with coloured boxes at the top of the figure; SNPs not part of the haplotypes are not marked with coloured boxes.

a

	+1752	+1843	+1846	+2066	+2077	+2225	+2381	+2458	+2772	+2838	+2919
Wild type	G	G	G	G	G	T	A	A	-	C	T
Mutant	A	A	A	A	T	C	G	C	G	G	G
3'UTR-hap1											
3'UTR-hap1a											
3'UTR-hap1b											
3'UTR-hap2											
3'UTR-hap3											
3'UTR-hap4											

b

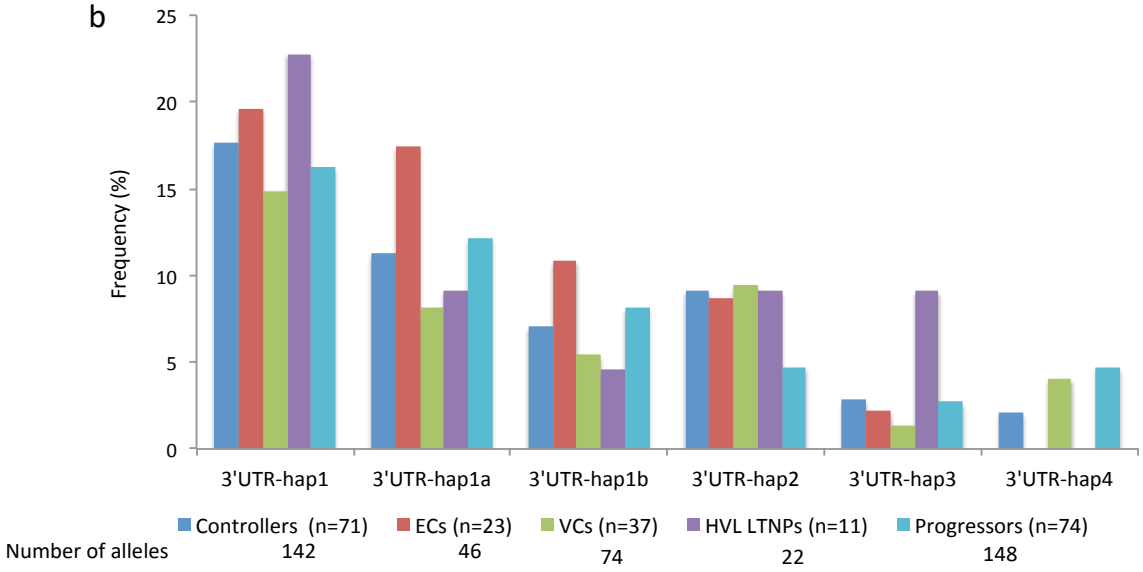


Figure 3.4 – a) CCR5 polymorphisms that form each haplotype. Colour shaded boxes show the respective SNPs or indels that form each haplotype and b) bar graph showing the allelic frequencies (%) of CCR5 3'UTR haplotypes in black South African controllers, controller subgroups (ECs, VCs and HVL LTNPs) and progressors. ECs: elite controllers, VCs: viraemic controllers, HVL LTNPs: high viral load long-term non-progressors.

3.3.3 Haplotypes spanning the *CCR5* 5'UTR and 3'UTR

Linkage disequilibrium between SNPs in the *CCR5* 5'UTR and the *CCR5* 3'UTR regions was investigated. Visual analysis and Haploview software revealed fourteen haplotypes spanning the *CCR5* 5'UTR and 3'UTR regions (Figure 3.5). Four of the fourteen haplotypes have previously been identified: HHA and +1752 G>A SNP, HHE and +2919 T>G SNP, HHF and +2919 T>G SNP, HHC and +2077 G>T SNP, in order of decreasing frequency (170).

			CCR5 5'UTR							*CCR5* 3'UTR										
			-2733	-2554	-2459	-2135	-2132	-2086	-1838	+1752	+1843	+1846	+2066	+2077	+2225	+2381	+2458	+2772	+2838	+2919
Promoter haplotype	**Associated 3'UTR SNPs**	**Wild type**	A	G	G	T	C	A	C	G	G	G	G	G	T	A	A	-	C	T
		Mutant	G	T	A	C	T	G	T	A	A	A	A	T	C	G	C	G	G	G
HHA	+1752																			
HHE	+2919																			
HHF	+2919																			
HHA	+1752 +1843 +1846																			
HHA	+1752 +1843 +1846 +2225																			
HHC	+2077																			
HHA	+1752 +1843 +1846 +2225 +2838																			
HHC	+2077 +2458																			
HHD	+1752																			
HHG	+2919																			
HHE	+2772 +2919																			
HHC	+1752 +2381																			
HHC	+2066 +2077 +2772																			
HHC	+2066 +2077																			

Figure 3.5 - Polymorphisms that form haplotypes spanning the *CCR5* 5'UTR and 3'UTR in black South Africans living with HIV-1 (progressors and controllers). Colour shaded boxes show the respective SNPs or indels that form each haplotype. 3'UTR SNPs that have linkage with the same root 5'UTR haplotype are in the same colour.

The +2919 T>G SNP showed strong LD with the HHE, HHF*1, HHF*2 and HHG*1 promoter haplotypes (r^2=0.73, D'=0.89). Comparison of individuals having both the HHE haplotype and the +2919 T>G SNP showed a significant association with HIV-1 progression when comparing progressors to VCs (p=0.011; OR=3.66; CI=1.36-9.84) and the total group of controllers (p=0.008; OR=2.61; CI=1.30-5.22). Since the +2919 T>G SNP is in LD with HHE, HHF and HHG, we also looked at the combinatorial effect of having these high expressing haplotypes together with the +2919 T>G SNP. The representation of individuals with either HHE and/or HHF and/or HHG*1 together with the 3'UTR +2919 T>G SNP was again overrepresented in progressors when compared to the total group of controllers (p=0.028; OR=1.80; CI=1.09-2.89), however had a less significant result than when looking at only the possession of both HHE and the +2919 T>G SNP.

When comparing controllers and progressors who possessed both HHE or HHF*1 or HHF*2 or HHG*1 and the +2772 -/G indel, the total controllers had significantly lower instances of this occurring (p=0.009; OR=6.67; CI=1.44-31.02). In addition, when comparing ECs + VCs to progressors as well as the total controller group to progressors, progressors were more likely to possess an HHE promoter haplotype, the 3'UTR +2772 insertion and the 3'UTR +2919 T>G SNP (p=0.042; OR=8.17; CI=1.00-66.43 and p=0.056; OR=4.77; CI=0.99-22.94, respectively). However, it is necessary to again note the large confidence intervals of these findings, suggesting that further study in larger cohorts is needed to determine true significance.

3.3.4 Potential markers for HIV-1 disease progression

The variants that were the strongest indicators of HIV-1 progression in our study were the 3'UTR +2919 T>G SNP in dominant mode (controllers vs progressors: p=0.001, $p_{bonferroni}$=0.016), heterozygosity for the 3'UTR +2919 T>G SNP (controllers vs progressors: p=0.002, $p_{bonferroni}$=0.032) and heterozygosity for 5'UTR-2SNP-hap (controllers vs progressors: p=0.003, $p_{bonferroni}$=0.048). A summary of all deleterious associations (controllers vs progressors) is shown in Figure 3.6.

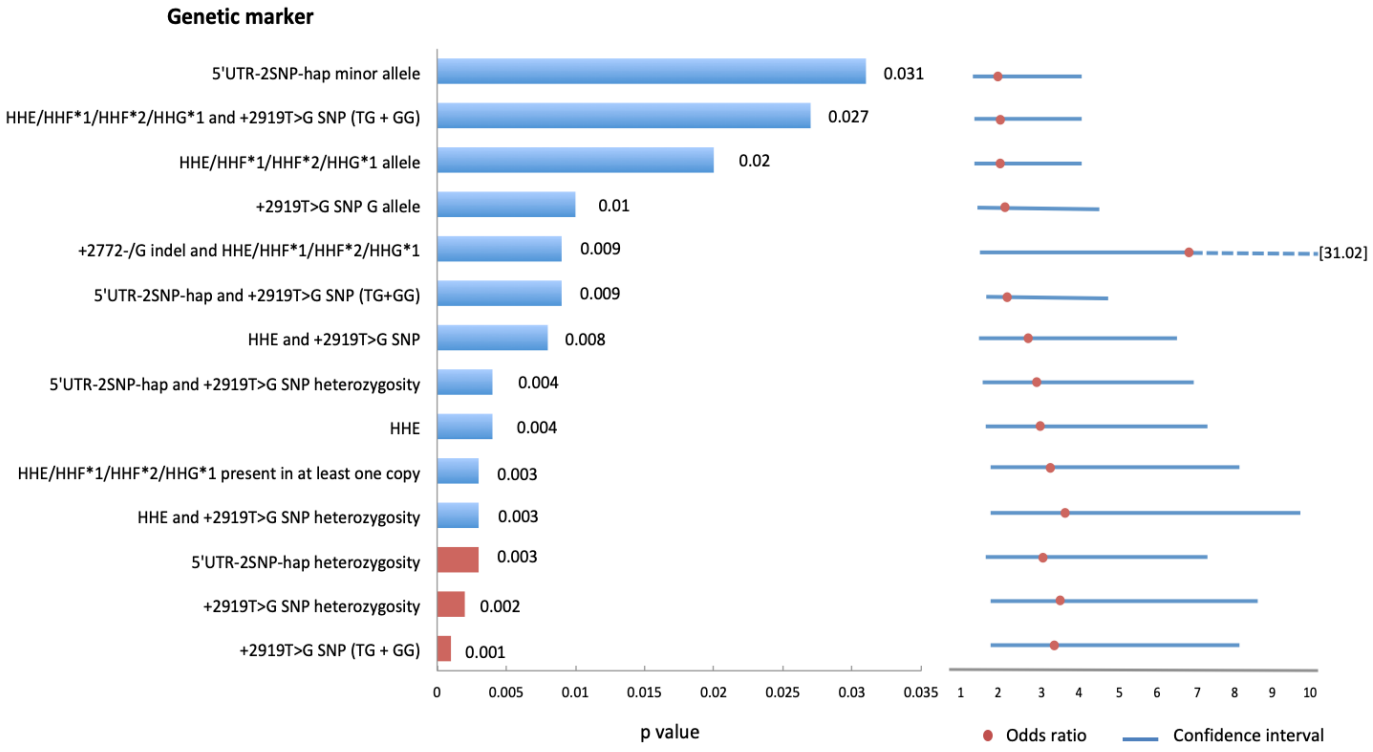


Figure 3.6 - Summary of the p values of all significant CCR5 genetic markers predictive of HIV-1 progression in black South Africans (HIV-1 controllers vs progressors). The smaller the p value is, the more predictive the biomarker. Red bars indicate p values that maintain significant Bonferroni correction. The blue lines indicate the confidence interval and the red circles indicate the odds ratio for the respective p values.

3.4 Discussion

In this study, we characterised polymorphisms (SNPs and indels) and intragenic haplotypes found within the *cis*-regulatory regions of *CCR5* in a group of HIV-1 controllers and progressors. Previously unreported polymorphisms and haplotypes have been identified and previously defined haplotypes within the *CCR5* gene have been expanded upon.

The -4223 C>T SNP, shown to disrupt the CpG-41 site and to date only found in southern Africans (115), is more prevalent in individuals with the *CCR5* HHA haplotype. Although the -4223 C>T SNP was associated with protection from HIV-1 acquisition in black South Africans in a previous study (115), associations did not reach statistical significance in that study or ours. In our study, all seven controllers with this SNP had at least one HHA haplotype. In the progressors, four of the five individuals possessing the -4223 T allele had at least one HHA haplotype, however, three of the five progressors possessing the -4223 T allele also had an HHE haplotype. Although this SNP was

not significantly overrepresented in controllers compared to progressors, taking into account that the HHE haplotype has reported high transcriptional promoter activity (112) and could therefore potentially negate the effect of the -4223 C>T SNP, removing the progressors with an HHE haplotype we considered a more informative comparison. It is also interesting to note that no ECs possessed the -4223 T allele, indicating that the -4223 C>T polymorphism may only be helpful in the presence of higher viraemia.

The distribution of *CCR5* gene promoter haplotypes is highly variable amongst ethnic groups. The various haplotypes have different promoter activity, correlating with differential cell surface CCR5 expression (109,112–115). Joshi et al., 2017 (112) calculated the relative promoter activity (RPA) of each *CCR5* 5'UTR haplotype *in vitro* using a luciferase-based assay and normalizing to the ancestral HHA haplotype. The combined relative promoter activity based on both alleles was also calculated. HHA, HHB, HHC and HHD had a low RPA (i.e., low expected CCR5 expression) whereas HHE, HHF and HHG had a high RPA (i.e., high expected CCR5 expression) (112).

The HHE haplotype has been described as deleterious in the context of HIV-1 control across multiple ethnicities and studies (116,171–173). In agreement, in this study, HHE was significantly underrepresented in the total group of controllers compared to progressors as well as in the VCs compared to the progressors. While the HHE haplotype has been identified as deleterious, homozygosity for HHE (HHE/HHE) was not shown to associate with disease-modifying effects in African Americans whereas in Caucasians, HHE homozygosity, but not HHE heterozygosity, associated with disease acceleration (116). Therefore, it is not only an individual CCR5 haplotype that has consequences on HIV-1 disease, but also the combinatorial (additive and/or interactive) effect of an individual's CCR5 genotype.

The CCR5 haplotype pair (genotype) in an individual and its relationship to the CCR5 levels on CD4+ T cells is partly related to whether one or both haplotypes have increased or decreased sensitivity to activation-associated demethylation (115). In our study, only one individual, a progressor, had the HHE/HHE genotype, indicating that it is a rare genotype in black South Africans and may not be contributing to HIV-1 disease progression or acquisition. Genotypes with at least one HHE haplotype, compared with genotypes lacking an HHE haplotype, have been associated with higher CCR5 levels (115).

In our study, HHA/HHC was significantly overrepresented in the total group of controllers compared to the progressors as well as in the VCs compared to the progressors. In African Americans, possession of an HHC haplotype has been associated with disease acceleration (116). However, if an HHC haplotype was paired with one of the haplotypes that was associated with protection in African Americans (HHA or HHF*2), the disease-accelerating effects of the HHC haplotype were negated, as can be seen in our black South African population, where the pairing of HHA (protective in African Americans) and HHC (deleterious in African Americans) i.e., the HHA/HHC genotype, was found to be protective (116). The HHA and HHC haplotypes have been reported to have reduced sensitivity to activation-associated demethylation (115), possibly revealing a functional cause as to why HHA/HHC may be protective in black South Africans. The HHD haplotype is found more commonly in ethnic African individuals. In a study on South African Caucasians and black South Africans, no HHD haplotypes were found in the Caucasian group, whereas HHD was the third most commonly found haplotype in the black South Africans after the HHA and HHE haplotypes (170). Possession of the HHD/HHG*1 genotype was found to be potentially beneficial in HIV-1 control in this study, specifically in viraemic controllers.

It is interesting to note that, while not significant, there was a complete absence of HHF*2, a relatively prevalent haplotype, in the HVL LTNP group. Since HHF*2 is defined by the presence of the *CCR2-V64I* variant, *CCR2* may be playing a role in control in this group. HVL LTNPs are a very unique group in adults (174–177) and to our knowledge, we have the largest cohort of such individuals worldwide (n=11). This makes it very difficult to verify the role of HHF*2 in this method of control of HIV-1 disease in different cohorts.

Several individual SNPs located in the *CCR5* promoter have been reported to affect the expression of CCR5. The −2459 G>A SNP (rs1799987) is in complete LD with the -2135 T>C SNP (rs1799988). The −2459 G>A SNP (and therefore the -2135 T>C SNP by association) has been linked to differences in CCR5 expression levels on CD14+ monocytes (178) and has been associated with the rate of progression to AIDS (117–119). In our study, the -2459 G>A and -2135 T>C SNPs (5'UTR-2SNP-hap) were significantly underrepresented in controllers compared to progressors. 5'UTR-2SNP-hap is common to the HHE, HHF and HHG haplotypes (which have been reported to be high promoter activity haplotypes (109,112)). Individuals heterozygous for 5'UTR-2SNP-hap were significantly less likely to control HIV-1 in total group comparisons as

well as in ECs and VCs compared to progressors and possession of the minor allele of 5'UTR-2SNP-hap was significantly underrepresented in controllers when compared to progressors. In a cohort of self-identified white and black patients, a study observed that black individuals on highly active anti-retroviral therapy (HAART) with the -2459 G allele achieved virologic success significantly earlier than individuals with the A allele, and that this association increased with stronger African ancestry (179). According to the 1000 Genomes Project (1000genomes.org), the minor allele frequency of this variant is 54% in Caucasians/Europeans and 40% in African populations.

The 3'UTR plays a major role in gene expression and regulation by influencing the localization, stability, export, and translation efficiency of an mRNA (180). The linkage patterns in the 3'UTR were complex, suggesting interesting selection pressures that are likely to have consequential functional implications that have not yet been elucidated. In the CCR5 3'UTR, the +2919 T>G SNP G allele was significantly underrepresented in controllers compared to progressors. Possession of the +2919 T>G SNP in the dominant mode was significantly associated with HIV-1 control. In addition, the +2919 TG genotype was significantly underrepresented in controllers when compared to progressors. In order to determine the prevalence of the 3'UTR +2919 T>G SNP in populations of sub-Saharan African descent, we used data available from the 1000 Genomes Project (1000genomes.org). Interestingly, the +2919 T>G SNP frequency in the controllers in our study strongly resembles that of the Yoruba population (minor allele frequency 31%), while the +2919 T>G SNP frequency in the progressors in our study more closely resembles that of the European/Caucasian population (minor allele frequency 53%), suggesting that the representation in controllers is more reflective of the background population and that the representation in progressors is skewed.

The +2919 T>G SNP was in strong LD with the CCR5 promoter HHE, HHF*1, HHF*2 and HHG*1 haplotypes and subsequently 5'UTR-2SNP-hap (common to these haplotypes), thus begging the question as to which polymorphism is functionally driving the deleterious effect on HIV-1 control in black South Africans. While possession of the HHE haplotype alone, the HHE haplotype with the +2919 T>G SNP, or any of the remaining three deleterious haplotypes (HHF*1, HHF*2, HHG*1) with the +2919 T>G SNP all significantly associated with HIV-1 progression ($p=0.004$; $p=0.003$, $p_{bonferroni}=0.048$; and $p=0.027$ respectively), there was a stronger association

with progression when looking at possession of the +2919 T>G SNP in the dominant mode alone (p=0.001. $p_{bonferroni}$=0.016). To our knowledge, no other studies have associated this SNP with increased risk of HIV-1 disease acquisition or more rapid progression of HIV-1 in any other population.

This study further emphasises the need for population specific studies with regards to potential genetic markers and HIV-1 control. Individuals in sub-Saharan Africa remain markedly understudied when compared with other populations around the world living with HIV-1. Overall, our results reproduce other studies with regards to the *CCR5* HHE haplotype being deleterious for HIV-1 disease progression. We found that the HHA haplotype and HHA/HHC genotype associated with protection from HIV-1 disease progression. We have characterised novel haplotypes in the 3'UTR and spanning the *CCR5* 5'UTR and 3'UTR. Our results suggest that two *CCR5* promoter SNPs (-2459 G>A and -2135 T>C) and one *CCR5* 3'UTR SNP (+2919 T>G) may be key functional variants with regards to HIV-1 control in black South Africans. It is important to note that only select comparisons remained significant after Bonferroni correction for multiple comparisons, thus future work including functional studies will help to reinforce our findings.

We propose that possession of the 3'UTR +2919 T>G SNP in the dominant mode, the strongest predictor for HIV-1 progression in this study (p=0.001, $p_{bonferroni}$=0.016), can be used as a marker for accelerated disease progression in black South Africans.

CHAPTER FOUR

4. CCR5 and CXCR6 expression in two ethnically distinct South African population groups

4.1 Introduction

The chemokine receptor CCR5 is an integral molecule with regards to both the acquisition and pathogenesis of HIV-1 (91,99,181). It is the main coreceptor that R5-tropic HIV-1 uses for entry into target cells (182). CCR5-using viruses are the predominant strains isolated from infected individuals during the early stages of viral infection and a majority of people living with HIV-1 harbour only CCR5-using viruses throughout the entire course of disease (183). CCR5 expression increases with HIV-1 disease progression and decreases after the initiation of antiretroviral therapy (184), further emphasizing its importance with regards to HIV-1. Inter-individual variation in CCR5 expression has been attributed to both genetic and environmental influences (185–187). A well-studied mutation in its homozygous form, *CCR5Δ32,* confers resistance to HIV-1 acquisition due to the subsequent lack of cell surface CCR5 protein, thus making CCR5 a target for a functional HIV-1 cure (188,189). This polymorphism is predominantly found in European populations and is essentially absent in African, East Asian, and American Indian populations (108).

CCR5 is not only a chemokine receptor but also a co-activation molecule expressed at the surface of T cells, thus CCR5 may be directly involved in the immune activation seen in individuals living with HIV-1 (190). Systemic immune activation is a feature of HIV-1 infection and increased T cell activation is an independent predictor of HIV-1 progression (191,192). The intensity of immune activation has been directly linked to the level of CCR5 expression in people living with HIV-1 (190). CCR5 density was found to correlate with, and be predictive of, the immune activation levels of HIV-1-infected individuals independently of HIV-1 viral load (190).

CXCR6, also known as Bonzo and STRL33, has been identified as a minor coreceptor used by HIV-1 to enter target cells in conjunction with CD4. While the chemokine receptor CXCR6 is considered a minor coreceptor for HIV-1, mediating the fusion of HIV-1 to $CD4^+$ T cells and increasing HIV-1 infectivity (193), CXCR6 acts as a major coreceptor in some SIV hosts, such as sooty mangabeys (SIVsmm), who exhibit non-pathogenic disease and an absence of disease progression (122,159). The preferential usage of CXCR6 over CCR5 by SIVsmm results in a lower pathogenicity of the virus as compared to HIV-1 (122,123). It has been proposed that usage of

CXCR6 for entry by SIVsmm, in addition to low CCR5 levels in these hosts, may result in virus being redirected to different cell targets compared to hosts with CCR5 as the main coreceptor, and that this differential targeting may promote infection of non-essential cells and limit infection of critical cells (122). Since HVL LTNPs exhibit a similar phenotype to SIV-infected sooty mangabeys (194), it could be postulated that CXCR6 may be contributing to disease control in these individuals. Additionally, select polymorphisms in *CXCR6* have been significantly associated with HIV-1 control in viraemic individuals (134–136). Given that some of these polymorphisms may be associated with altered CXCR6 expression (134,135,138), a role for CXCR6 expression in HIV-1 control in HVL LTNPs is plausible and worthy of investigation.

Ethnicity is an important variable with regards to varying expression levels of chemokine receptors (116,179,187,195). Both historic and recent environmental selective pressures contribute to ethnic genetic divergence (196). Thus, it is likely that populations will differ with regards to receptor expression levels. Ethnicity has been implicated in HIV-1 disease progression, with Gag-specific proliferation of both CD4+ and CD8+ T cells having been more frequently observed in black individuals compared to individuals from other ethnic groups (197,198). African Americans have also been shown to have a faster progression to AIDS and a shorter survival time from acquisition compared to other ethnic groups (199). Greater genetic diversity within the *CCR5* gene has been shown in black individuals compared to white, showing 39 mutations exclusive to black individuals (200).

The *CXCR6*-3K single nucleotide polymorphism (SNP) is highly prevalent in African Americans (44%), and is extremely rare in European populations (<1%) (136). Ethnic and population differences exist with regards to both the level of variability and the frequency of select variants and haplotype structures in a large number of genes, including *CXCR6* (135,201–203). Similarly to *CCR5* genetics, variation within the *CXCR6* gene was found to be greater in black South Africans compared to white South Africans (135).

Significant progress has been made regarding the role of the chemokine receptor CCR5 in connection with HIV-1 natural control (99). However, to further understand the mechanisms contributing to varying CCR5 expression levels and the role of CCR5 and CXCR6 in HIV-1 control, it is necessary to investigate the expression of these receptors in various cell types without the confounding effect of HIV-1 disease. To our knowledge, no studies have described constitutive

background expression of CXCR6 in an African context. Furthermore, the relationship between CCR5 and CXCR6 on the same cell types has not to our knowledge been studied.

This study was therefore conducted to investigate CCR5 and CXCR6 expression in the absence of HIV-1 disease in two ethnic population groups in South Africa. We compared background, constitutive CCR5 and CXCR6 expression on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells from both black and white healthy HIV-1-uninfected South Africans. We also examined associations between activated cells and CCR5 and CXCR6 expression, assessed the ratio of CCR5 to CXCR6 expression and lastly, correlated CCR5 and CXCR6 expression, with the aim of further understanding the role of these two important receptors.

4.2 Materials and Methods

4.2.1 Sample population

Refer to Chapter 2, Materials and Methods section 2.1. Briefly, healthy HIV-1-uninfected South African individuals (black [n=17] and white [n=21]) were recruited and were age- and sex-matched.

4.2.2 Flow cytometry

CCR5 and CXCR6 expression on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells was determined as described in Chapter 2, Materials and Methods section 2.7 and assessed as described in Materials and Methods section 2.9.

4.2.3 Statistical analyses

Statistical analyses were performed as described in Chapter 2, Materials and Methods section 2.10.

4.3 Results

4.3.1 CCR5 and CXCR6 expression differences

The frequency (percentage of CCR5/CXCR6-expressing cells) and density (CCR5/CXCR6 molecules per cell) of CCR5 and CXCR6 expression was assessed on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells from whole blood samples from study participants. The gating strategies are shown in Chapter 2, Figures 2.5 - 2.9.

In the total group (black and white individuals combined), for CCR5, CD8+ T cells had the highest frequency of CCR5-expressing cells (median: 31.3%), followed by $CD4^+$ T cells (median: 12.9%), NK cells (median: 11.6%), and $CD14^+$ monocytes (median: 3.7%), with CD19+ B cells having the lowest frequency of CCR5-expressing cells (median: 1.3%) (CD8 > CD4 > NK > CD14 > CD19) (Figure 4.1a). CD8+ T cells also had the highest density of CCR5 expression (median: 1130.7 molecules/cell), followed by CD4+ T cells (median: 753.8 molecules/cell), CD14+ monocytes (median: 613.4 molecules/cell), CD19+ B cells (median: 554.1 molecules/cell) and NK cells had the lowest CCR5 density of subsets studied (median: 384.4 molecules/cell) (CD8 > CD4 > CD14 > CD19 > NK) (Figure 4.1b).

For CXCR6, CD8+ T cells had the highest frequency of CXCR6-expressing cells (median: 9.2%), followed by NK cells (median: 3.9%), CD4+ T cells (median: 1.9%), and CD19+ B cells (median: 0.7%), with CD14+ monocytes having the lowest frequency of CXCR6-expressing cells (median: 0.5%) (CD8 > NK > CD4 > CD19 > CD14) (Figure 4.1a). CD14+ monocytes had the highest density of CXCR6 expression (median: 1528.9 molecules/cell), followed by CD4+ T cells (median: 880.0 molecules/cell) and CD8+ T cells (median: 828.8 molecules/cell), NK cells (median: 813.9 molecules/cell) and CD19+ B cells (median: 456.0 molecules/cell) (CD14 > CD4 > CD8 > NK > CD19) (Figure 4.1b).

Comparison of CCR5 expression between the black and white population groups showed that CCR5 density was significantly lower on CD8+ T cells of black individuals compared to white individuals ($p=0.014$). No differences were seen between populations with regards to the frequency of CCR5-expressing cells. CXCR6 expression did not differ significantly in frequency or density between the black and white population groups on any of the cell subsets analysed.

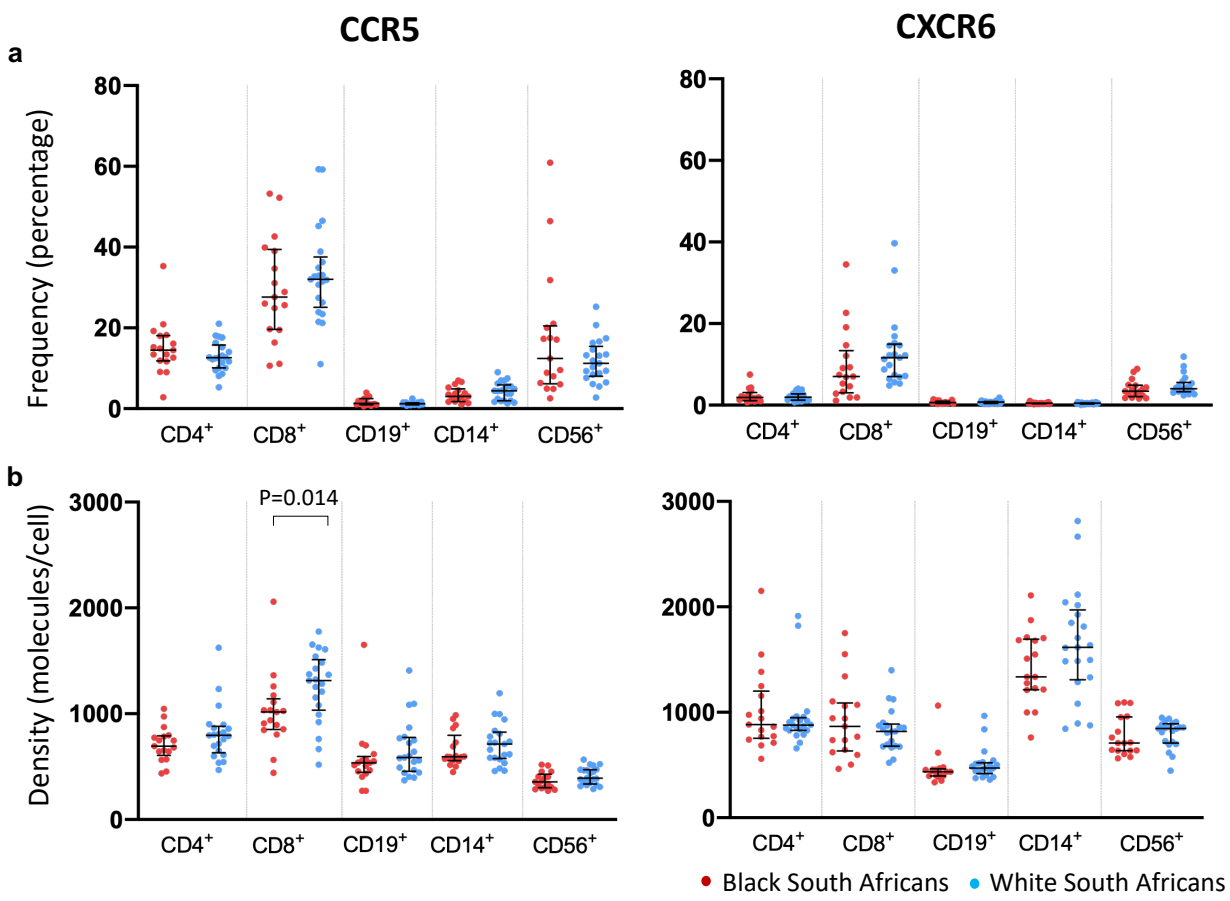


Figure 4.1 - Graphs showing CCR5 (left) and CXCR6 (right) expression on lymphocyte subsets in black and white individuals. The frequency (a) and density (b) of CCR5 and CXCR6 expression is shown on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ natural killer cells. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

4.3.2 T cell memory subset proportions differ between black and white South Africans

The proportions of CD4+ and CD8+ memory T cell subsets for black and white South Africans were assessed and compared (Figure 4.2a). Black individuals had a strong trend towards a lower proportion of naïve CD4+ T cells compared to white individuals (p=0.051; Figure 4.2a). Black individuals had a significantly higher proportion of terminally differentiated CD8+ T cells compared to white individuals (p=0.024; Figure 4.2b).

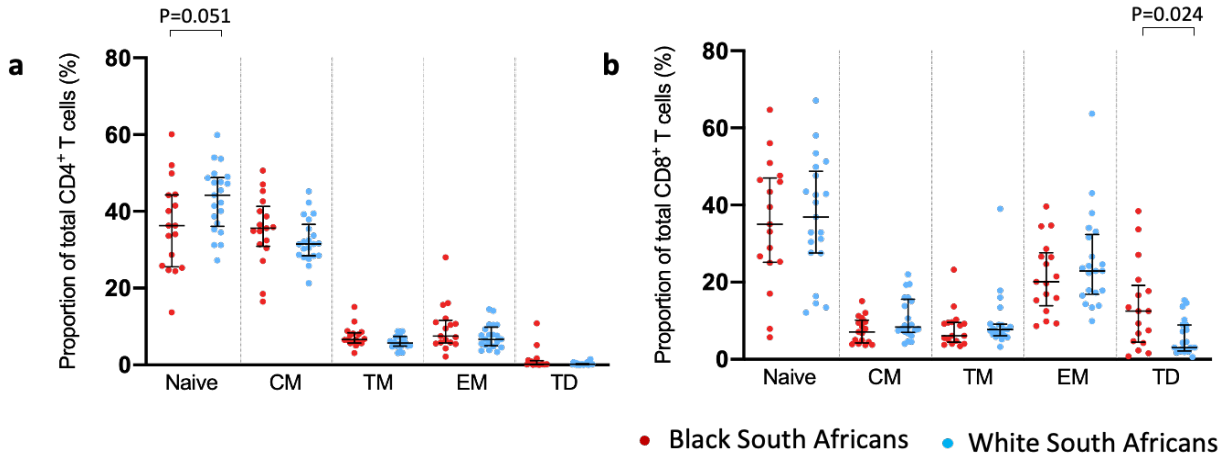


Figure 4.2 - Graphs showing T cell subset proportions of (a) total CD4+ T cells and (b) total CD8+ T cells in black and white South Africans. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles. CM: central memory, TM: transitional memory, EM: effector memory, TD: terminally differentiated.

4.3.3 CCR5 and CXCR6 expression on cell subsets

CCR5 expression was next analysed on phenotypically and functionally different subsets of CD4+ and CD8+ T cells (naïve, central memory [CM], transitional memory [TM], effector memory [EM], terminally differentiated [TD]), monocytes ($CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$, $CD14^{+}CD16^{bright}$) and NK cells ($CD56^{bright}$, $CD56^{dim}$, $CD56^{+}CD16^{+}$, $CD56^{bright}CD16^{+}$, $CD56^{dim}CD16^{+}$, $CD56^{bright}CD16$, $CD56^{dim}CD16^{-}$).

CD4+ and CD8+ T cell naïve and memory subsets were defined based on the differential expression of CD45RO, CCR7 and CD62L (Chapter 2, Figures 2.5 and 2.6). Monocytes were divided into classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical subsets ($CD14^{+}CD16^{bright}$) according to differing CD14 and CD16 expression (Chapter 2, Figure 2.8). CD56+ NK cells were divided into seven different subsets based on the relative expression of CD56 and CD16 (Chapter 2, Figure 2.9).

4.3.3.1 CD4+ T cells

The frequency of CCR5-expressing cells, as well as CCR5 density, increased with progressive CD4+ T cell differentiation up to effector memory CD4+ T cells in both population groups, and

decreased in terminally differentiated CD4+ T cells (Figure 4.3a). CCR5 was minimally expressed (percentage and density) on naïve CD4+ T cells, increasing on central memory CD4+ T cells and then transitional memory CD4+ T cells, with effector memory CD4+ T cells having the highest frequency of CCR5-expressing cells, and transitional memory and effector memory CD4+ T cells having the highest CCR5 density (frequency: N < CM < TM < EM > TD; density N < CM < TM ~ EM > TD). The pattern of expression for CXCR6 was similar to CCR5 (Figure 4.3).

While no differences were seen between groups with regards to CCR5 frequency, black individuals had significantly lower CCR5 density on naïve and central memory CD4+ T cells compared to white individuals (p=0.048 and p=0.018, respectively; Figure 4.3b). CXCR6 expression did not differ significantly in frequency or density between black and white individuals on any CD4+ T cell subset (Figure 4.3).

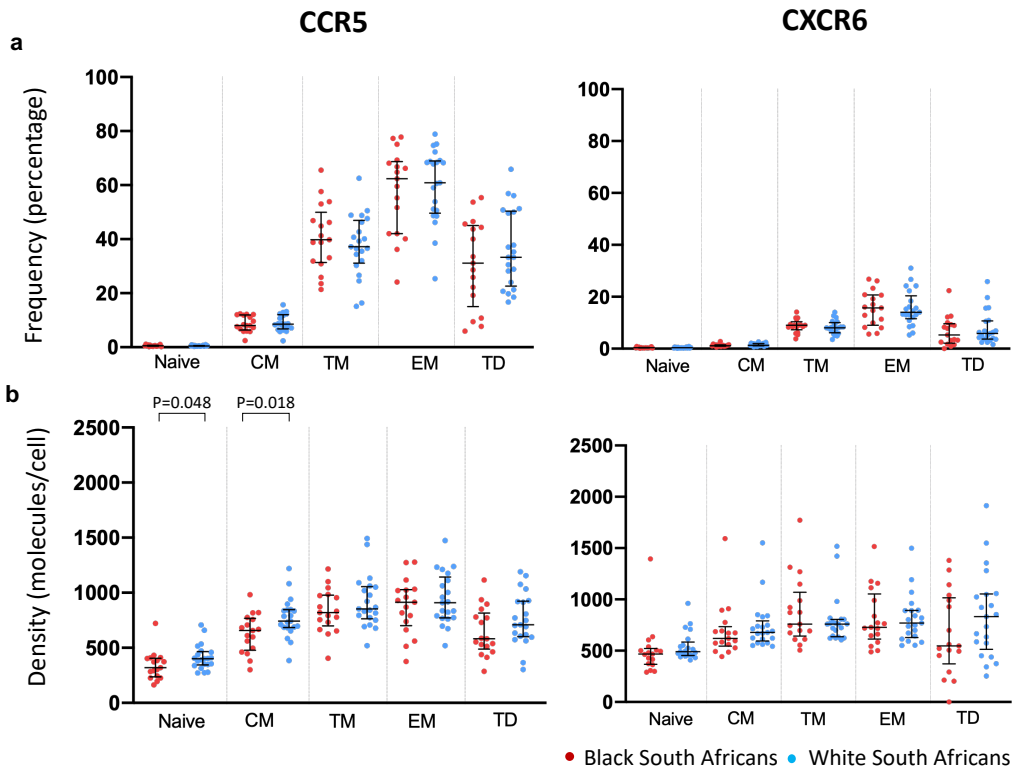


Figure 4.3 - Graphs showing CCR5 (left) and CXCR6 (right) expression on CD4+ T lymphocyte subsets in black and white South African individuals. The (a) frequency and (b) density of CCR5 and CXCR6 expression is shown on naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD4+ T cell subsets. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

4.3.3.2 CD8+ T cells

Similarly to that observed for CD4+ T cells, the frequency of CCR5-expressing cells and CCR5 density differed substantially in the naïve and memory CD8+ T cell subsets (N < CM < TM ~ EM > TD; Figure 4.4a). The frequency of CXCR6-expressing CD8+ T cells also increased with progressive CD8+ T cell differentiation in both population groups. In the black population group, naïve CD8+ T cells had the lowest expression of CXCR6, followed by central memory CD8+ T cells and then transitional memory CD8+ T cells, while effector memory and transitional memory cells had similar CXCR6 expression (N < CM < TM ~ EM > TD; Figure 4.4a). No significant differences were seen between groups with regards to CCR5 expression. The frequency of CXCR6-expressing naïve CD8+ T cells and effector memory CD8+ T cells was significantly lower in black compared to white individuals (p=0.031 and p=0.029, respectively; Figure 4.4). CXCR6 density did not differ significantly between black and white individuals on any CD8+ T cell subset.

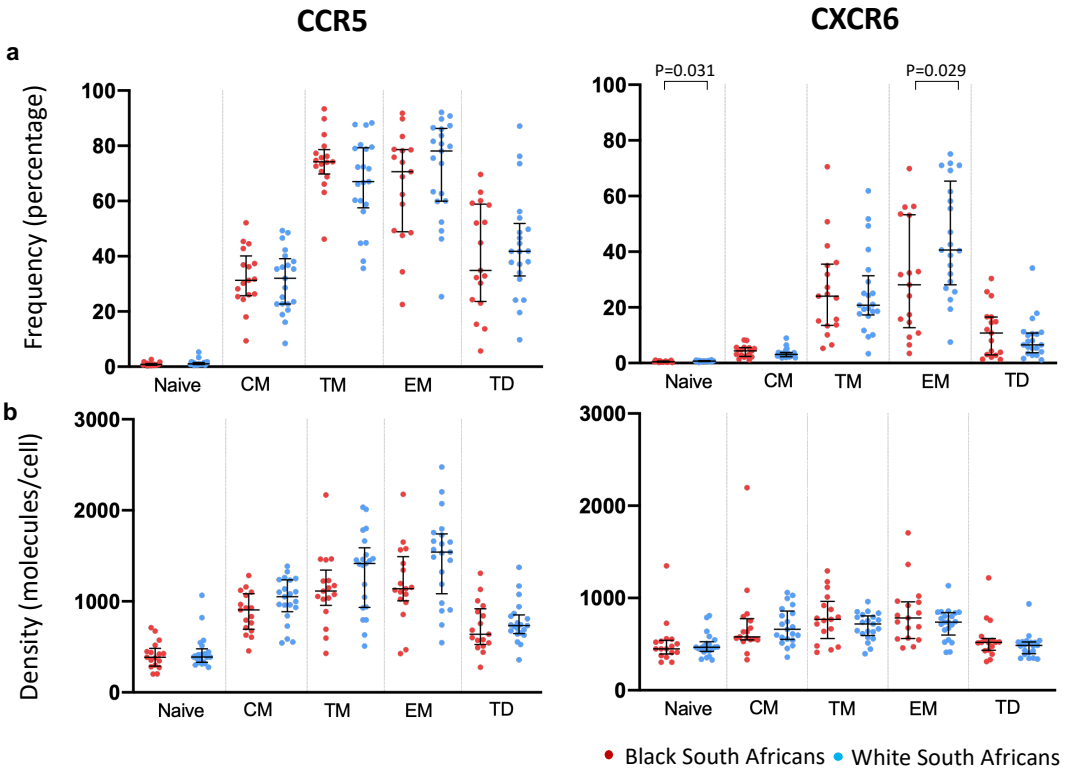


Figure 4.4 – Graphs showing CCR5 (left) and CXCR6 (right) expression on CD8+ T lymphocyte subsets in black and white South African individuals. The (a) frequency and (b) density of CCR5 and CXCR6 expression is shown on naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

4.3.3.3 Monocytes

In both population groups, compared to the $CD14^{+}CD16^{+}$ intermediate and $CD14^{+}CD16^{bright}$ non-classical subsets, the $CD14^{+}CD16^{-}$ classical monocytes had the lowest frequency of CCR5-expressing cells (Figure 4.5a). CCR5 density was, however, similar on all three monocyte subsets across population groups (Figure 4.5b). With regards to CXCR6 expression, in both population groups, the $CD14^{+}CD16^{-}$ monocytes had the lowest frequency of CXCR6-expressing cells, followed by the $CD14^{+}CD16^{bright}$ monocytes, with the $CD14^{+}CD16^{+}$ monocytes having the highest frequency of CXCR6-expressing cells (Figure 4.5a). CXCR6 density was similar on all three monocyte subsets across population groups (Figure 4.5b).

The only significant difference between groups with regards to CCR5 expression was a lower frequency of CCR5-expressing $CD14^{+}CD16^{bright}$ non-classical monocytes in black individuals compared to white individuals ($p=0.015$; Figure 4.5a). The frequency of CXCR6-expressing $CD14^{+}CD16^{+}$ monocytes was significantly higher in the black population than in the white population group ($p=0.003$; Figure 4.5b).

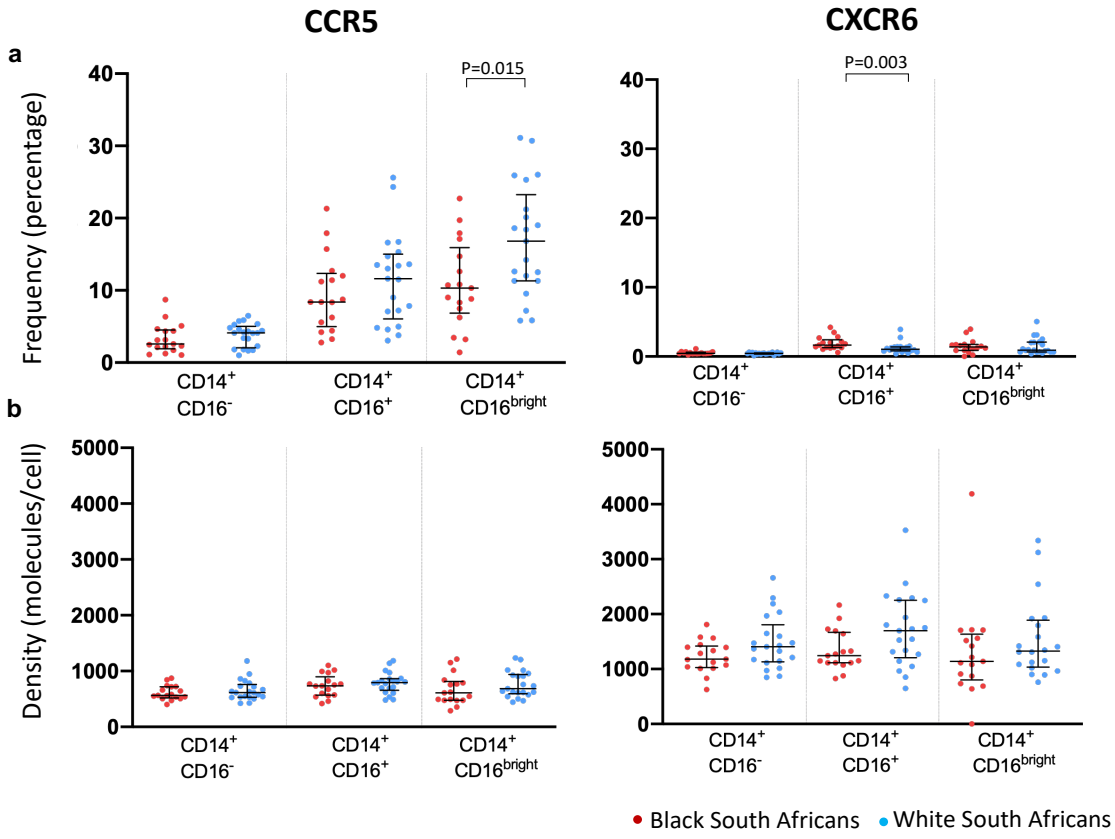


Figure 4.5 – Graphs showing CCR5 (left) and CXCR6 (right) expression on monocyte subsets in black and white South African individuals. The (a) frequency and (b) density of CCR5 and CXCR6 expression is shown on $CD14^{+}CD16^{-}$ classical monocytes, $CD14^{+}CD16^{+}$ intermediate monocytes and $CD14^{+}CD16^{bright}$ non-classical monocytes. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

4.3.3.4 NK cells

In the combined group, CCR5 had a higher expression (both frequency and density) on CD16- vs CD16+ NK cell subsets ($p<0.0001$ for both $CD56^{bright}CD16^{-}$ vs $CD56^{bright}CD16^{+}$ and $CD56^{dim}CD16^{-}$ vs $CD56^{dim}CD16^{+}$ comparisons). Only the frequency of CCR5-expressing $CD56^{bright}$ and $CD56^{bright}CD16^{+}$ NK cells were higher than on the $CD56^{dim}$ counterparts ($p<0.0001$ for both comparisons; Table 4.1).

In the combined group, CXCR6 expression (frequency and density) was higher on CD16- vs CD16+ NK cell subsets and $CD56^{bright}$ vs $CD56^{dim}$ subsets (CCR5 density on $CD56^{bright}CD16^{+}$ vs $CD56^{dim}CD16^{+}$ $p=0.007$; $p<0.0001$ for all other comparisons; Table 4.1). The $CD56^{bright}CD16^{-}$ NK cell subset thus had the highest frequency and density of CXCR6 expression.

Black individuals had a significantly higher frequency of CCR5-expressing $CD56^{bright}CD16^{+}$ NK cells and a significantly lower frequency of CCR5-expressing $CD56^{dim}CD16^{-}$ NK cells compared to white individuals (p=0.033 and p=0.011, respectively; Figure 4.6a). There were no significant differences in the frequency of CXCR6-expressing NK cells or CCR5 and CXCR6 density on any subset between the two population groups.

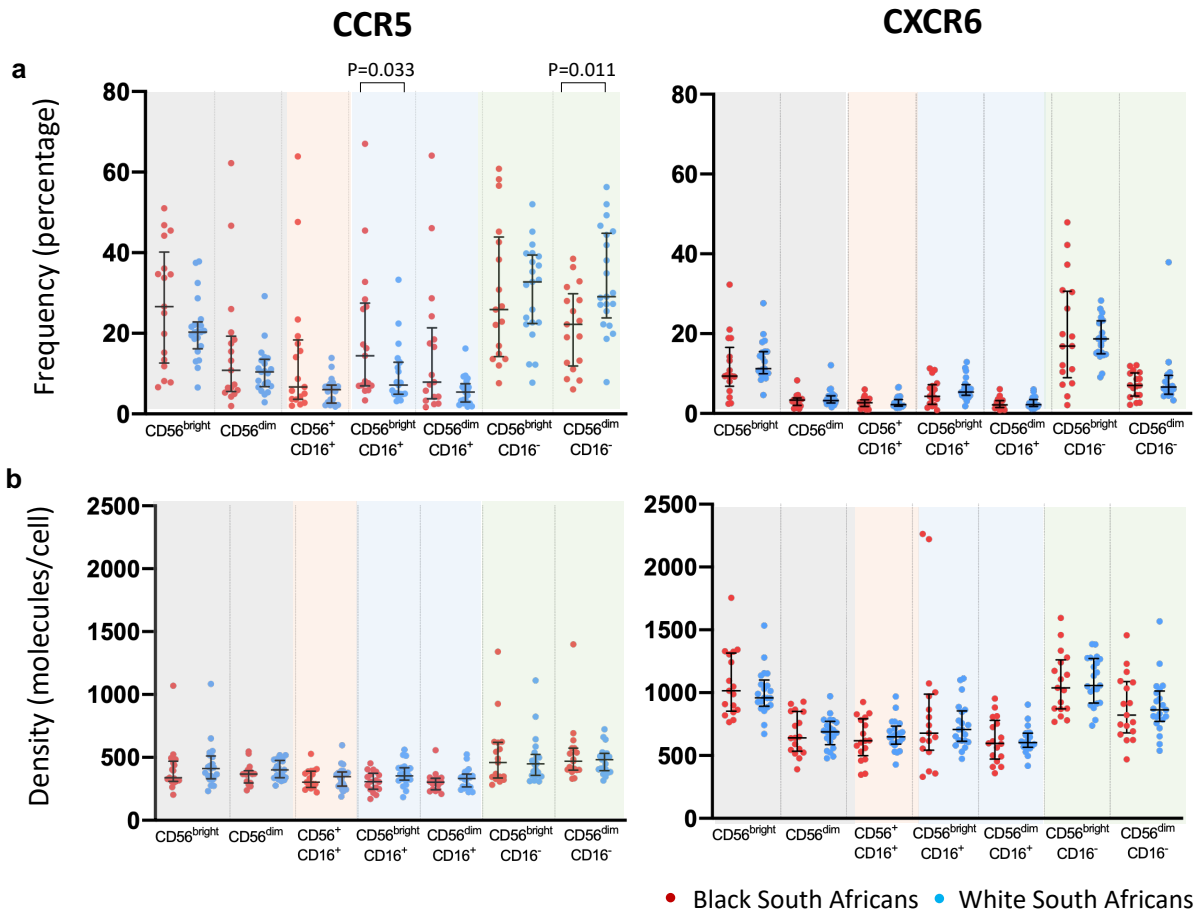


Figure 4.6 - Graphs showing CCR5 (left) and CXCR6 (right) expression on NK cell subsets in black and white South African individuals. The (a) frequency and (b) density of CCR5 and CXCR6 expression is shown on NK cell subsets. Purple shading depicts the $CD56^{bright}$ and $CD56^{dim}$ subsets. Orange shading depicts the total $CD56^{+}CD16^{+}$ subset. Blue shading depicts the CD16+ subsets. Green shading depicts the CD16- subsets. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

Table 4.1 - Comparison of CCR5 and CXCR6 expression (frequency and density) on $CD56^{bright}$ versus $CD56^{dim}$ and CD16+ versus CD16- subsets of NK cells for the total group.

CCR5

NK cell subset	Frequency (median %)	NK cell subset	Frequency (median %)	p value
$CD56^{bright}$	**20.5**	$CD56^{dim}$	10.4	<0.0001
$CD56^{bright}CD16^{+}$	**7.9**	$CD56^{dim}CD16^{+}$	6.0	<0.0001
$CD56^{bright}CD16^{-}$	26.7	$CD56^{dim}CD16^{-}$	27.2	0.28
$CD56^{bright}CD16^{-}$	**26.7**	$CD56^{bright}CD16^{+}$	7.9	<0.0001
$CD56^{dim}CD16^{-}$	**27.2**	$CD56^{dim}CD16^{+}$	6.0	<0.0001

NK cell subset	Density (median molecules/cell)	NK cell subset	Density (median molecules/cell)	p value
$CD56^{bright}$	394.7	$CD56^{dim}$	368.4	0.06
$CD56^{bright}CD16^{+}$	324.2	$CD56^{dim}CD16^{+}$	321.0	0.19
$CD56^{bright}CD16^{-}$	459.5	$CD56^{dim}CD16^{-}$	483.0	0.73
$CD56^{bright}CD16^{-}$	**459.5**	$CD56^{bright}CD16^{+}$	324.2	<0.0001
$CD56^{dim}CD16^{-}$	**483.0**	$CD56^{dim}CD16^{+}$	321.0	<0.0001

CXCR6

NK cell subset	Frequency (median %)	NK cell subset	Frequency (median %)	p value
$CD56^{bright}$	**10,8**	$CD56^{dim}$	3,3	<0.0001
$CD56^{bright}CD16^{+}$	**5,3**	$CD56^{dim}CD16^{+}$	2,2	<0.0001
$CD56^{bright}CD16^{-}$	**18,1**	$CD56^{dim}CD16^{-}$	6,8	<0.0001
$CD56^{bright}CD16^{-}$	**18,1**	$CD56^{bright}CD16^{+}$	5,3	<0.0001
$CD56^{dim}CD16^{-}$	**6,8**	$CD56^{dim}CD16^{+}$	2,2	<0.0001

NK cell subset	Density (median molecules/cell)	NK cell subset	Density (median molecules/cell)	p value
$CD56^{bright}$	**989,3**	$CD56^{dim}$	672,2	<0.0001
$CD56^{bright}CD16^{+}$	**693,5**	$CD56^{dim}CD16^{+}$	599,4	0,007
$CD56^{bright}CD16^{-}$	**1053,9**	$CD56^{dim}CD16^{-}$	847,6	<0.0001
$CD56^{bright}CD16^{-}$	**1053,9**	$CD56^{bright}CD16^{+}$	693,5	<0.0001
$CD56^{dim}CD16^{-}$	**847,6**	$CD56^{dim}CD16^{+}$	599,4	<0.0001

Orange shading: $CD56^{bright}$ subset. Green shading: $CD56^{dim}$ subset. Blue shading: CD16- subset. Yellow shading: CD16+ subset. Where comparisons are significant, the higher value is bolded.

4.3.4 Ratios and correlations: CCR5 and CXCR6 expression

The potential relationship between CCR5 and CXCR6 expression on cells was investigated. The ratio of CCR5 to CXCR6 expression for each individual was calculated and compared between groups (Figures 4.7 and 4.8) and the correlation of CCR5 and CXCR6 expression on each cell type was investigated (Table 4.2).

4.3.4.1 Ratio of CCR5 to CXCR6 expression

When assessing gross cell subsets, the ratios of CCR5 to CXCR6 expression did not differ significantly between population groups (Figure 4.7).

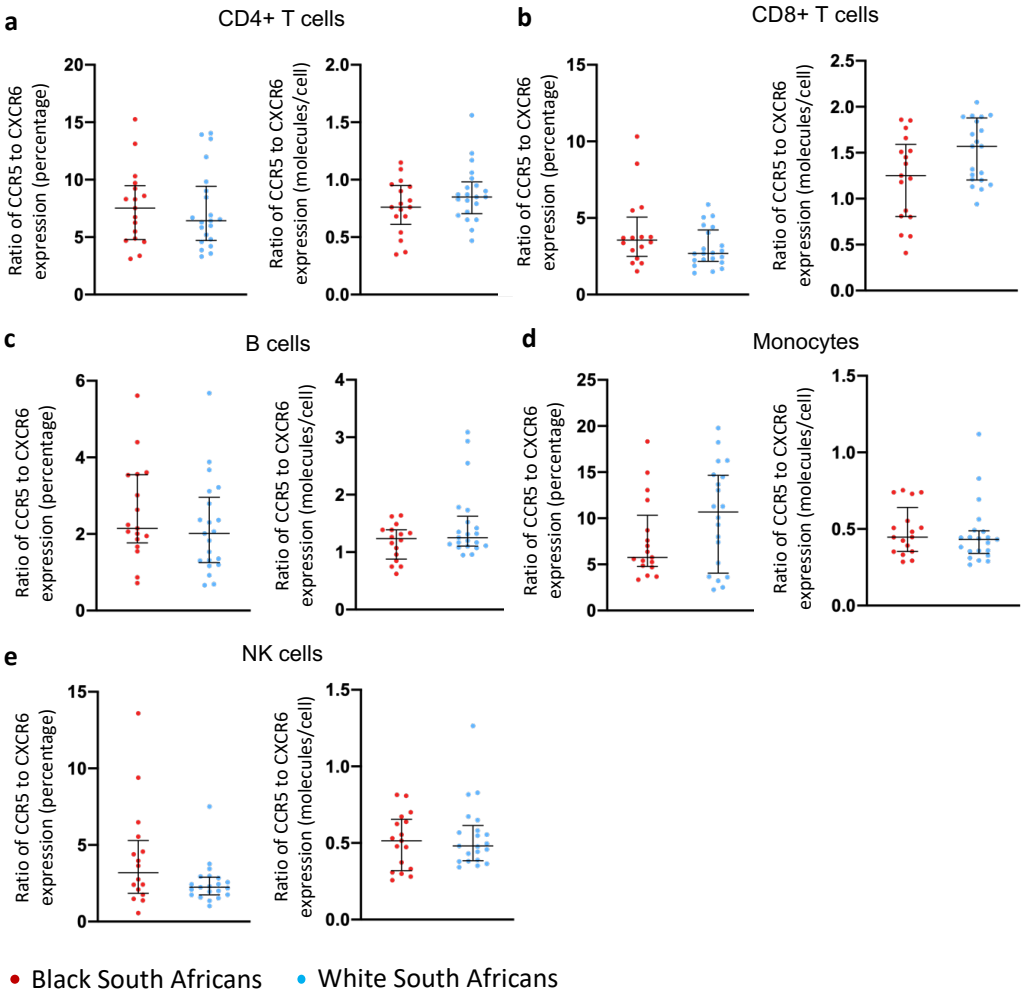


Figure 4.7 - Graphs showing the ratio of frequency (left) and density (right) of CCR5 to CXCR6 expression for the gross subset of (a) CD4+ T cells, (b) CD8+ T cells, (c) B cells, (d) monocytes and (e) NK cells. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

However, when investigating the ratio of CCR5 to CXCR6 expression on cell subsets, significant findings emerged (Figure 4.8). The black population had more individuals with a higher ratio of CCR5 to CXCR6-expressing effector memory CD8+ T cells (p=0.035) and a lower ratio of CCR5 to CXCR6 density on transitional memory and effector memory $CD8^+$ T cells compared to the white population (p=0.024 and p=0.004, respectively; Figure 4.8b). The black population had more individuals with a lower ratio of CCR5 to CXCR6-expressing $CD14^+CD16^+$ and $CD14^+CD16^{bright}$ monocytes compared to the white population (p=0.045 and p=0.008, respectively; Figure 4.8c) and had more individuals with a higher ratio of CCR5 to CXCR6 expressing $CD56^{bright}CD16^+$ NK cells compared to the white population (p=0.0004; Figure 4.8d).

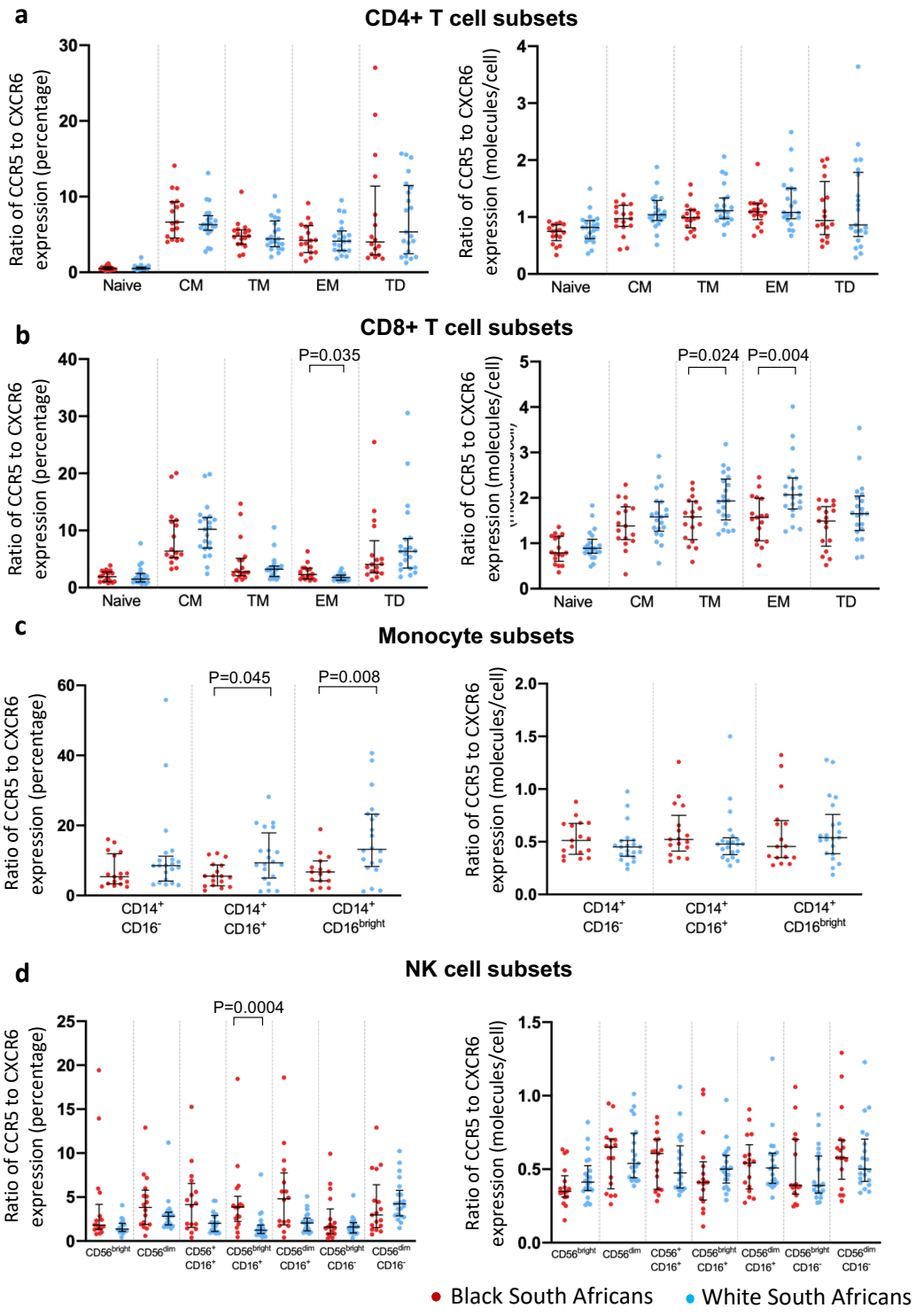


Figure 4.8 - Graphs showing the ratio of frequency (left) and density (right) of CCR5 to CXCR6 expression for the (a) CD4+ T cell subsets, (b) CD8+ T cell subsets, (c) monocyte subsets and (d) NK cell subsets. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles. CM: central memory, TM: transitional memory, EM: effector memory, TD: terminally differentiated.

4.3.4.2 Correlation between CCR5 and CXCR6 expression

CCR5 and CXCR6 expression (frequency and density) on all cell types was correlated to assess the potential relationship between the two receptors. Results of these analyses are shown in Table 4.2.

On CD4+ T cells, the percentage of CCR5-expressing cells significantly positively correlated with the percentage of CXCR6-expressing total, central memory and transitional memory CD4+ T cells. Additionally, there were very strong positive correlations between CCR5 and CXCR6 density on all CD4+ T cell subsets except for terminally differentiated CD4+ T cells. The percentage of CCR5-expressing cells positively correlated with the percentage of CXCR6-expressing total, naïve, effector memory and terminally differentiated CD8+ T cell subsets and CCR5 density positively correlated with CXCR6 density on naïve, transitional memory and effector memory CD8+ T cells. On B cells, only CCR5 and CXCR6 density positively correlated. On NK cells, the percentage of CCR5-expressing cells positively correlated with the percentage of CXCR6-expressing $CD56^{+}CD16^{+}$ NK cells and CCR5 density positively correlated with CXCR6 density on $CD56^{bright}CD16^{+}$ NK cells (refer to Table 4.2).

Table 4.2 - Correlation analyses results for CCR5 versus CXCR6 expression on CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells.

	CCR5 vs CXCR6			
	Frequency		Density	
Cell type	**R value**	**p value**	**R value**	**p value**
Total CD4+ T cells	0.636	<0.0001	0.487	0.002
Naïve	0.296	0.071	0.536	0.0005
CM	0.435	0.006	0.541	0.0005
TM	0.355	0.029	0.518	0.0009
EM	0.225	0.174	0.502	0.001
TD	0.167	0.317	0.291	0.077
Total CD8+ T cells	0.69	<0.0001	0.311	0.057
Naïve	0.393	0.015	0.38	0.019
CM	0.186	0.268	0.229	0.166
TM	0.295	0.072	0.372	0.022
EM	0.807	<0.0001	0.371	0.022
TD	0.58	<0.0001	0.268	0.104
Total B cells	0.268	0.104	0.501	0.001
Total Monocytes	0.186	0.262	0.247	0.136
$CD14^{+}CD16^{-}$	-0.056	0.737	0.141	0.4
$CD14^{+}CD16^{+}$	-0.084	0.617	0.294	0.073
$CD14^{+}CD16^{bright}$	-0.114	0.494	0.288	0.08
Total NK cells	0.264	0.109	-0.199	0.23
$CD56^{bright}$	0.153	0.358	0.144	0.389
$CD56^{dim}$	0.299	0.068	-0.109	0.516
$CD56^{+}CD16^{+}$	0.424	0.008	0.101	0.547
$CD56^{bright}CD16^{+}$	0.226	0.173	0.402	0.012
$CD56^{dim}CD16^{+}$	0.342	0.065	0.201	0.226
$CD56^{bright}CD16^{-}$	-0.105	0.532	-0.002	0.991
$CD56^{dim}CD16^{-}$	0.222	0.181	-0.027	0.873

Significant results highlighted in red. CM: central memory, TM: transitional memory, EM: effector memory, TD: terminally differentiated.

4.3.5 Activated cells, ethnicity and CCR5 and CXCR6 expression

HIV-1 disease progression is linked to increased expression of activation markers and activated CD4+ T cells have been shown to have elevated levels of CCR5 expression (204,205). CCR5 and CXCR6 expression were assessed to determine if there was an increase in expression on activated cells in our population groups, using HLA-DR as a marker for activation. HLA-DR is a marker for activation on T cells and NK cells (206,207). HLA-DR is constitutively expressed on B cell and monocytes. HLA-DR on monocytes is upregulated in HIV-infection and is most expressed on the intermediate subset (208).

It was initially determined if the percentage of HLA-DR+ cells differed between black and white South Africans on the gross cell subsets studied (Figure 4.9). Black South Africans had a significantly lower percentage of HLA-DR+ CD4+ T cells compared to white South Africans ($p=0.008$; Figure 4.9a). CCR5 and CXCR6 expression on HLA-DR+ and HLA-DR- cells was subsequently compared for all cell subsets (Figure 4.10).

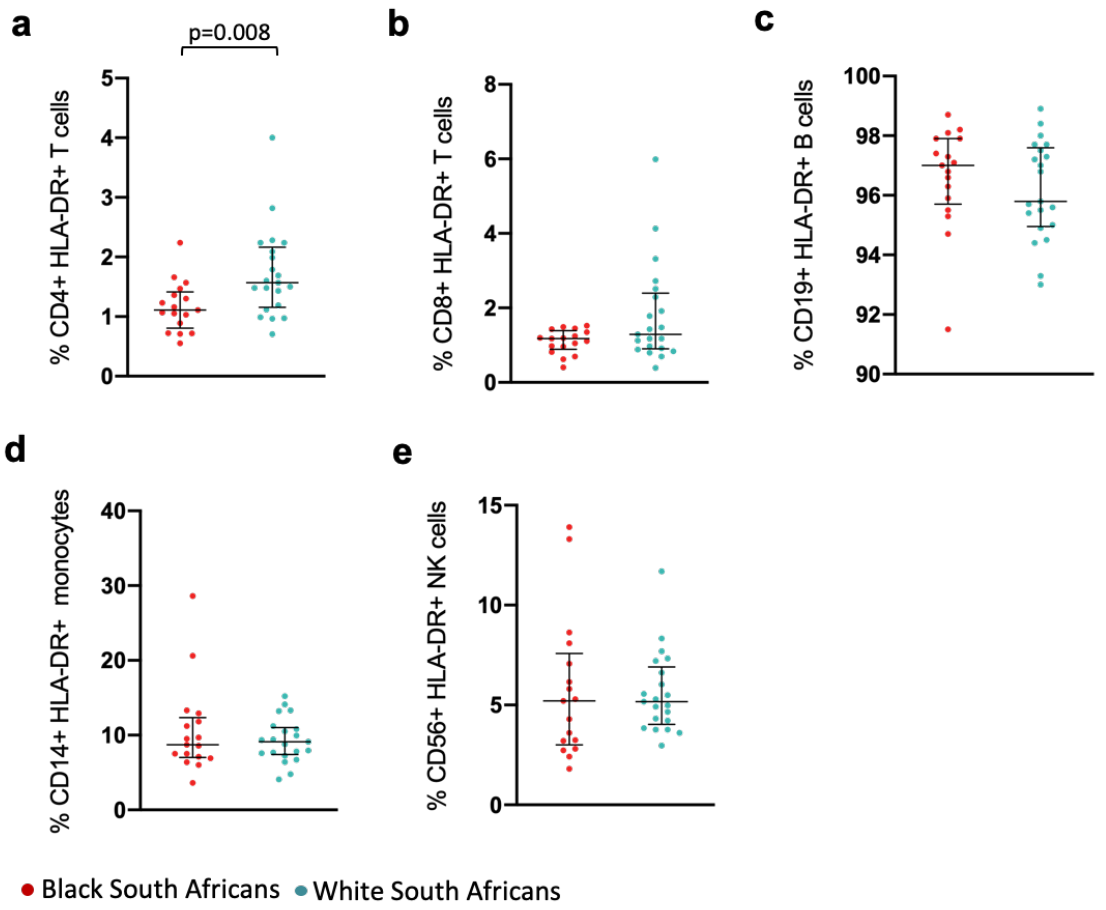


Figure 4.9 - Graphs showing the percentage of HLA-DR+ (a) CD4+ T cells, (b) CD8+ T cells, (c) B cells, (d) monocytes and (e) NK cells in black and white South Africans. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

4.3.5.1 Expression differences on HLA-DR+ versus HLA-DR- cells

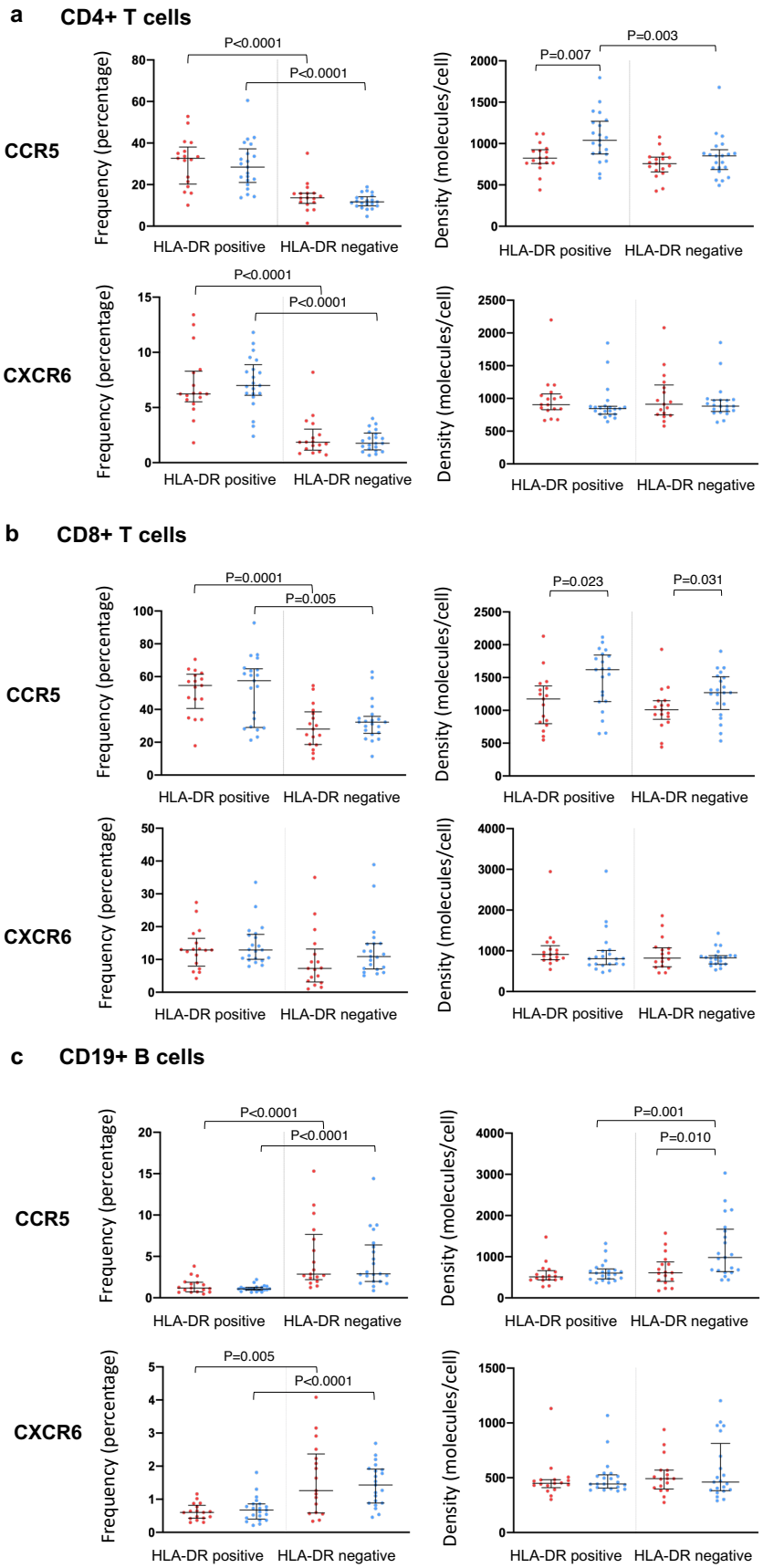
The frequency of CCR5-expressing CD4+ T cells, CD8+ T cells, CD14+ monocytes and CD56+ NK cells was significantly higher among HLA-DR+ compared to HLA-DR- cells in both the black and white population groups ($p<0.0001$ for all comparisons except CD8+ T cells - black: $p=0.0001$, white: $p=0.005$). The frequency of CXCR6-expressing CD4+ T cells, CD14+ monocytes and CD56+ NK cells was significantly higher in HLA-DR+ compared to HLA-DR- cells in both the black and white population groups ($p<0.0001$ for CD4+ T cell and monocyte comparisons, $p=0.045$ [black] and $p=0.029$ [white] for NK cell comparison – refer to Figure 4.10). The frequency of CXCR6-expressing CD8+ T cells showed no difference between HLA-DR+ and HLA-DR- subsets.

CCR5 density was significantly higher on HLA-DR+ compared to HLA-DR- CD4+ T cells only in white individuals ($p=0.003$; Figure 4.10a), CD14+ monocytes (black: $p=0.0001$, white: $p<0.0001$; Figure 4.10d) and NK cells ($p<0.0001$ for both population groups; Figure 4.10e). CXCR6 density did not differ between HLA-DR+ and HLA-DR- cells on any cell subset.

The percentage of CCR5- and CXCR6-expressing B cells was lower among HLA-DR+ compared to HLA-DR- cells for both population groups ($p<0.0001$ for all comparisons except CXCR6 black individuals [$p=0.005$]; Figure 4.10c). CCR5 density was significantly lower on HLA-DR+ B cells compared to HLA-DR- B cells in white individuals only ($p=0.002$).

4.3.5.2 Expression differences between population groups

Black individuals had significantly lower CCR5 density on HLA-DR+ CD4+ T cells compared to white individuals ($p=0.007$) and had significantly lower CCR5 density on HLA-DR+ and HLA-DR- CD8+ T cells compared to white individuals ($p=0.023$ and $p=0.031$, respectively; Figure 4.10b). When assessing B cells, black individuals had significantly lower CCR5 density on HLA-DR- B cells compared to white individuals ($p=0.010$; Figure 4.10c). Results from monocytes showed that compared with white individuals, black individuals had a significantly lower percentage of CCR5-expressing HLA-DR- CD14+ monocytes ($p=0.046$) and significantly lower CCR5 and CXCR6 density on HLA-DR+ monocytes compared to white individuals ($p=0.045$ and $p=0.009$, respectively; Figure 4.10d).


a
CD4+ T cells
CCR5
CXCR6
Frequency (percentage)
Density (molecules/cell)
P<0.0001
P<0.0001
P=0.007
P=0.003
HLA-DR positive
HLA-DR negative
b
CD8+ T cells
P=0.0001
P=0.005
P=0.023
P=0.031
c
CD19+ B cells
P=0.001
P=0.010

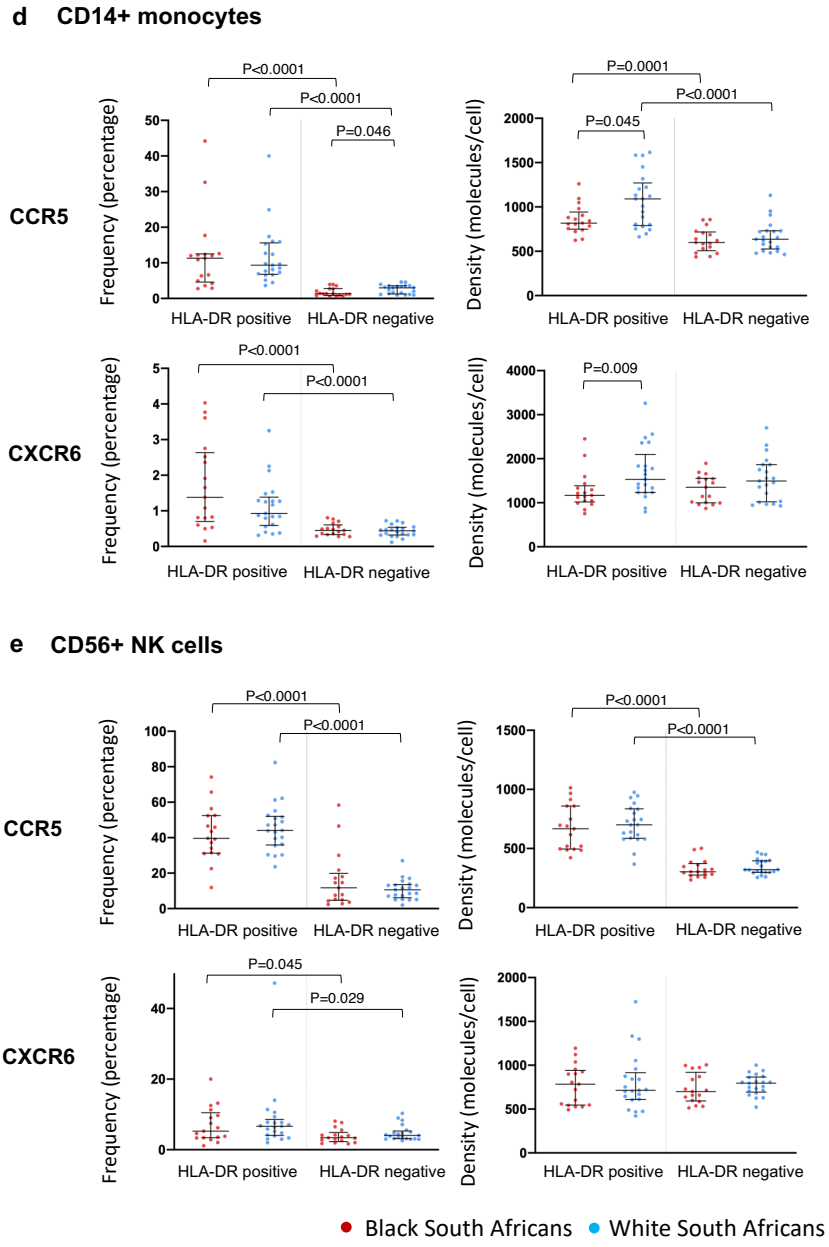


Figure 4.10 - CCR5 and CXCR6 expression on HLA-DR^{+} and HLA-DR^{-} lymphocyte subsets in black and white South African individuals. The frequency (left column) and density (right column) of CCR5 and CXCR6 expression is shown on HLA-DR+ and HLA-DR- (a) CD4+ T cells, (b) CD8+ T cells, (c) CD19+ B cells, (d) CD14+ monocytes and (e) CD56+ natural killer cells. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles.

4.3.5.3 Activated CD4+ and CD8+ T cells correlated with CCR5 and CXCR6 expression

To assess the relationship between activation (measured by HLA-DR percentage expression on both CD4+ and CD8+ T cells) and CCR5 and CXCR6 expression (both as percentage-expressing cells and density), these variables were correlated. Results are depicted as heatmaps in Figure 4.11 and Figure 4.12. It is interesting to note, upon visual inspection of the heatmaps, that the two populations showed differences in correlations (positive vs negative) in a number of cell subset comparisons.

With regards to CCR5 expression, HLA-DR expressing CD4+ T cells positively correlated with percentage of CCR5-expressing naïve CD8+ T cells in black individuals and in the total group (R=0.542, p=0.025 and R=0.381, p=0.018, respectively) and negatively correlated with percentage of CCR5-expressing $CD56^{bright}$ NK cells in black individuals (R=-0.498, p=0.042; Figure 4.11). HLA-DR expressing CD4+ T cells positively correlated with CCR5 density on transitional memory CD4+ T cells in white individuals (R=0.452, p=0.040) and negatively correlated with CCR5 density on effector memory CD4+ T cells in black individuals (R=-0.551, p=0.022). HLA-DR expressing CD4+ T cells positively correlated with CCR5 density on total CD56+ NK cells (white: R=0.444, p=0.044; total group: R= 0.438, p=0.006), $CD56^{dim}$ NK cells (total group: R=0.430, p=0.007), $CD56^{+}CD16^{+}$ NK cells (white: R=0.476, p=0.029) and $CD56^{dim}CD16^{+}$ NK cells (white: R=0.553, p=0.009; total group: R=0.381, p=0.018).

Significant negative correlations were observed between HLA-DR+ CD4+ T cells and the percentage of CXCR6-expressing CD14+CD16+ monocytes in the total group (R=-0.351, p=0.031) and CXCR6 density on terminally differentiated CD8+ T cells and total CD56+ NK cells in black individuals (R=-0.483, p=0.049 and R=-0.586, p=0.013, respectively) and $CD14^{+}CD16^{bright}$ monocytes in white individuals (R=-0.561, p=0.008). Refer to Figure 4.11.

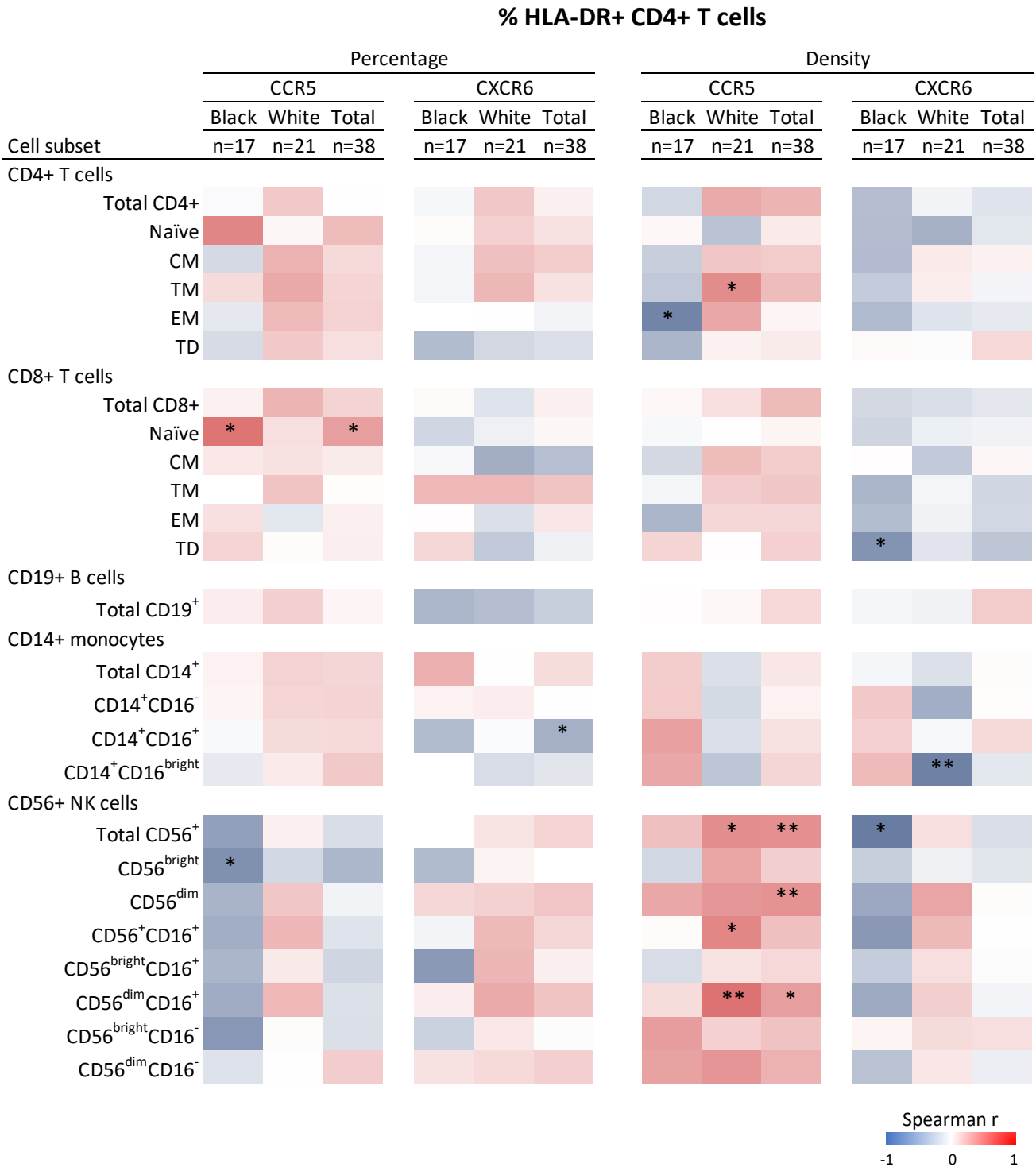


Figure 4.11 - Heatmap showing correlations between % HLA-DR+ CD4+ T cells and CCR5 and CXCR6 expression on CD4+ T cells and subsets CD8+ T cells and subsets, CD19+ B cells, CD14+ monocytes and subsets and CD56+ NK cells and cell subsets. Correlations are shown for both frequency of expression (left 2 columns) and density (right 2 columns). The darker the colour, the stronger the correlation. * p<0.05; ** p<0.01; *** p<0.001. CM: central memory, TM: transitional memory, EM: effector memory, TD: terminally differentiated.

HLA-DR expressing CD8+ T cells positively correlated with percentage of CCR5-expressing naïve CD4+ T cells (R=0.495, p=0.043), naïve CD8+ T cells (black: R=0.498, p=0.042; total group: R=0.332, p=0.041) and terminally differentiated CD8+ T cells in black individuals (R=0.630, p=0.007; Figure 4.12). HLA-DR expressing CD8+ T cells also positively correlated with CCR5 density on total CD4+ T cells (white: R=0.466, p=0.033; total group: R=0.405, p=0.012) and transitional memory CD4+ T cells in white individuals (R=0.522, p=0.015). In black individuals only, HLA-DR expressing CD8+ T cells positively correlated with CCR5 density on CD14+ monocytes (R=0.495, p=0.043), $CD14^{+}CD16^{+}$ monocytes (R=0.544, p=0.024) and $CD14^{+}CD16^{bright}$ monocytes (R=0.591, p=0.013). HLA-DR expressing CD8+ T cells also positively correlated with CCR5 density on total CD56+ NK cells (white: R=0.515, p=0.017; total group: R=0.406, p=0.011), $CD56^{bright}$ NK cells (white: R=0.524, p=0.015), $CD56^{dim}$ NK cells (total group: R=0.343, p=0.035) and $CD56^{dim}CD16^{+}$ (white: R=0.490, p=0.024).

With regards to CXCR6 expression, HLA-DR expressing CD8+ T cells negatively correlated with percentage of CXCR6-expressing terminally differentiated CD4+ T cells (white: R=-0.463, p=0.035; total group: R=-0.327, p=0.045) and B cells in black individuals only (R=-0.608, p=0.010). HLA-DR expressing CD8+ T cells positively correlated with percentage of CXCR6-expressing $CD56^{bright}CD16^{+}$ NK cells (white: R=0.437, p=0.048) and $CD56^{dim}CD16^{+}$ NK cells (white: R=0.537, p=0.012; total group: R=0.405, p=0.012). In white individuals, HLA-DR expressing CD8+ T cells negatively correlated with CXCR6 density on $CD14^{+}CD16^{bright}$ monocytes (R=-0.445, p=0.043) and positively correlated with CXCR6 density on $CD56^{dim}$ (R=0.551, p=0.010) and $CD56^{+}CD16^{+}$ NK cells (R=0.505, p=0.019). Refer to Figure 4.12.

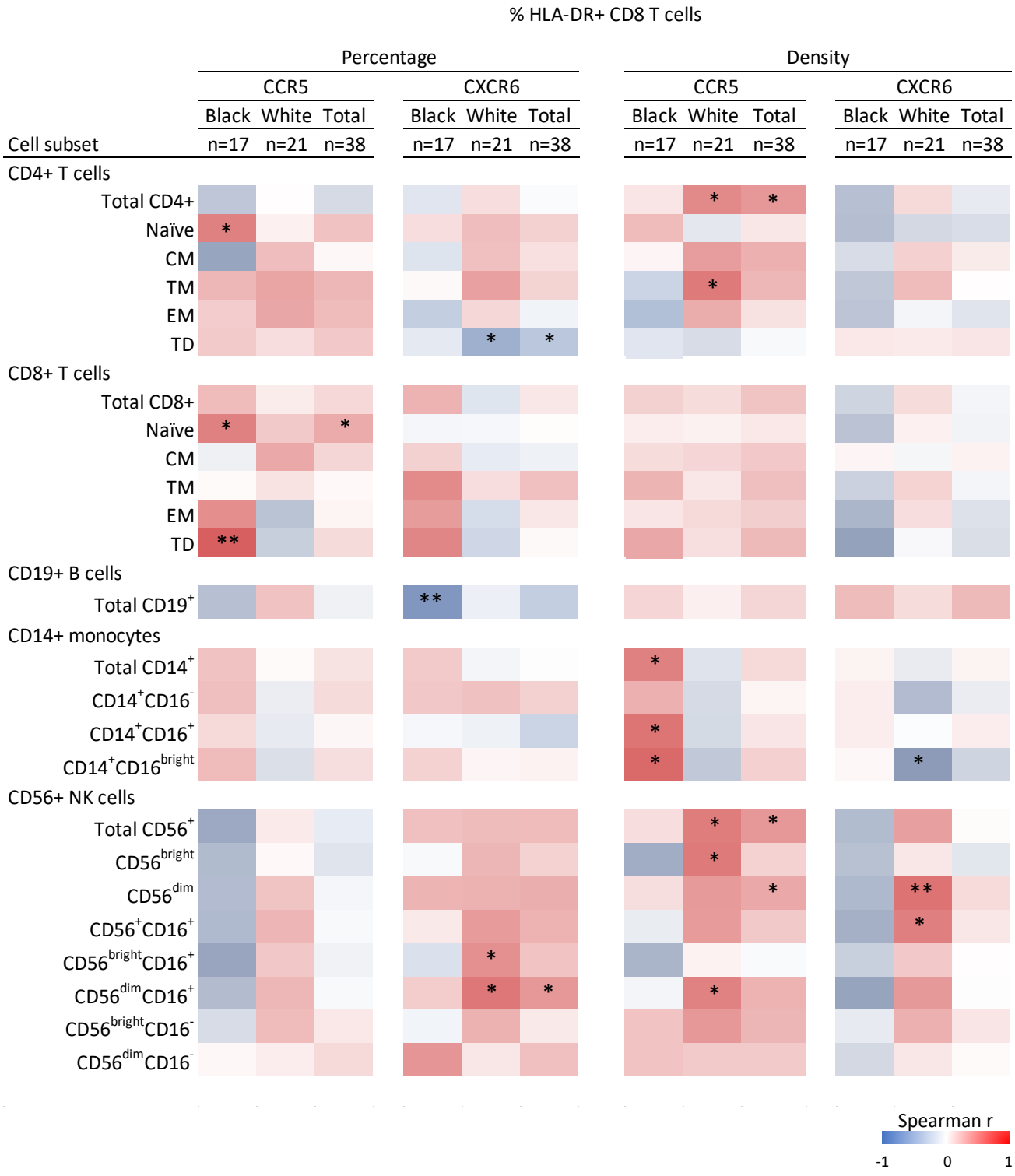


Figure 4.12 - Heatmap showing correlations between % HLA-DR+ CD8+ T cells and CCR5 and CXCR6 expression on CD4+ T cells and subsets CD8+ T cells and subsets, CD19+ B cells, CD14+ monocytes and subsets and CD56+ NK cells and cell subsets. Correlations are shown for both frequency of expression (left 2 columns) and density (right 2 columns). The darker the colour, the stronger the correlation. * p<0.05; ** p<0.01; *** p<0.001. CM: central memory, TM: transitional memory, EM: effector memory, TD: terminally differentiated.

4.3.6 Summary of findings

The majority of significant findings from this portion of the study showed a lower expression of both CCR5 and CXCR6 in black individuals compared to white individuals. Interestingly, CXCR6 density did not differ significantly between groups on any cell type. A summary of all significant population-specific expression differences and correlations of CCR5 and CXCR6 expression is shown in Figure 4.13. We did not perform corrections for multiple comparisons because of our small sample size. Therefore, significant results may fall away post correction. Studies with bigger sample sizes will be beneficial in determining true significance.

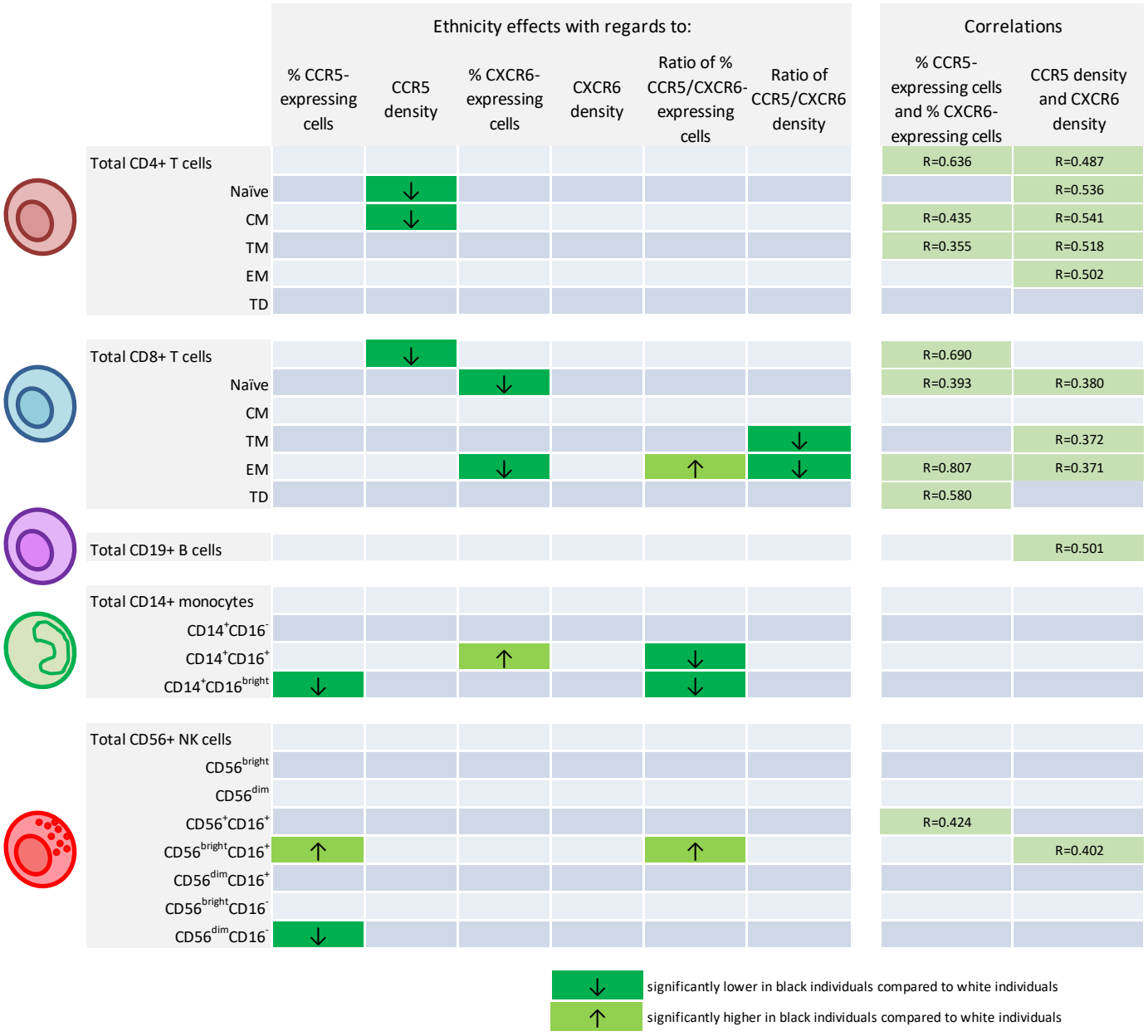


Figure 4.13 - Summary of significant ethnic differences with regards to CCR5 and CXCR6 expression, ratios of CCR5 to CXCR6 expression and correlations of CCR5 and CXCR6 expression on CD4+ T cell subsets, CD8+ T cell subsets, B cells, monocyte subsets and NK cell subsets. R values are shown for significant correlations. CM: central memory, TM: transitional memory, EM: effector memory, TD: terminally differentiated.

4.4 Discussion

CCR5 and CXCR6 expression (frequency and density) was assessed in black and white HIV-1-uninfected individuals with South African ancestry to provide important reference knowledge with regards to potential population-specific differences in HIV-1 control.

Here we found significant differences in CCR5 and CXCR6 expression between healthy black and white South African individuals, a relationship between CCR5 and CXCR6 expression on certain cell subsets, and correlations between HLA-DR+ CD4+ and CD8+ T cells and CCR5 and CXCR6 expression. This work builds on a previous study from our research group (195) which found striking differences in both cell activation and CCR5 expression between South African black and white healthy controls. In this study, a different healthy cohort was assessed and both CXCR6 expression and CCR5 expression was investigated on CD4+ and CD8+ T cells and subsets, CD19+ B cells, CD14+ monocytes and subsets and CD56+ NK cells and subsets.

CCR5 expression impacts HIV-1 acquisition and disease progression in multiple ways; it influences cell entry and infection as an HIV-1 coreceptor and also functions as an important immune modulatory protein (209,210). Low CCR5 expression levels correlate with a reduced ability to infect T cells with macrophage-tropic HIV-1, *in vitro* (95,105). Central memory CD4+ T cells with high CCR5 density associated with a rapid progression of HIV-1 (100). Increased CCR5 density at the surface of CD4+ T cells was shown to associate with higher viral loads and faster disease progression in HIV-1-infected persons and had an effect on the post-entry efficiency of R5 HIV-1 infection (104). While nearly all HIV-1 strains use CCR5 and/or CXCR4, the low pathogenic SIVsmm strain uses CXCR6 a major coreceptor (122–124).

Expression of CXCR6 has been detected on a variety of cells including natural killer (NK), dendritic, activated T cells and NKT cells (125,126). CXCR6 is a marker for effector T cells (127,128) and promotes homing of lymphocytes to extra-lymphoid tissue (129). CXCR6 and its ligand CXCL16 play an important role in T-cell trafficking and cell to cell contact during inflammation (130).

Emphasizing the importance of population-specific studies, we found multiple significant differences in constitutive CCR5 and CXCR6 expression on various cell types between black and white South Africans.

T cells

Prior to evaluating CCR5 and CXCR6 expression on T cell subsets, differences in proportions of T cell subsets between black and white South Africans was investigated. There was a trend towards a significantly smaller proportion of naïve CD4+ T cells in black individuals compared to white individuals. A significantly higher proportion of terminally differentiated CD8+ T cells was seen in black individuals compared to white individuals. This could potentially result in different population-specific immune responses.

When analysing gross cell subsets, black and white individuals differed significantly with regards to CCR5 density on CD8+ T cells. This is in agreement with a previous study from our group assessing a different cohort of South African healthy controls where black South Africans had a significantly lower CCR5 density on $CD8^+$ T cells compared to white South Africans (195). Assessment of CCR5 expression on naïve and memory T cell subsets showed that the percentage of both CCR5-expressing CD4+ and CD8+ T cells increased with progressive T cell differentiation in both the black and white population groups, which is in line with previously reported findings where the differentiation state of T cells was shown to influence CCR5 levels (95,211,212). CCR5 expression was higher on memory subsets compared to naïve subsets in our study. This finding concurs with other studies that have shown that among memory cells, CCR5 expression was higher on effector memory compared to central memory T cells (95,211).

While we did not see a difference between populations with regards to CCR5 expression on total CD4+ T cells (a finding also seen in a previous study from our research group (195), when evaluating expression on CD4+ T cell naïve and memory subsets, some distinct differences were seen. There was a significantly lower density of CCR5 on naïve and central memory CD4+ T cells in black individuals compared to white individuals.

In both population groups, there was a higher frequency of CXCR6-expressing CD8+ T cells compared to CD4+ T cells. This is in agreement with studies that investigated CXCR6 expression on both human and sooty mangabey T cells (123,126,130). The frequency of both CXCR6-expressing $CD4^+$ and $CD8^+$ T cells increased with progressive T cell differentiation in both the black and white population groups, which is in line with previously reported findings where CXCR6 expression was shown to be minimal on naïve subsets with almost exclusive expression

on CD45RO^{+} memory subsets (123,126,213). While no differences in CCR5 expression on CD8+ T cell subsets were seen with regards to ethnicity, we found significant differences in CXCR6 expression between the two population groups. In our study, there was a significantly lower frequency of CXCR6-expressing naïve and effector memory CD8+ T cells in black individuals compared to white individuals.

The ratio of individuals' CCR5 to CXCR6 expression differed between population groups on CD8+ T cell subsets, monocyte subsets and NK cell subsets. It is interesting that both density and frequency of CCR5 expression significantly positively correlated with CXCR6 expression on multiple subsets of CD4+ and CD8+ T cells. This provides insight into the possible relationship between the two receptors. CC and CXC chemokines have previously been shown to synergise, increasing leukocyte recruitment to areas of inflammation (214). Synergistic interactions between chemokines may contribute to the enhancement and modification of inflammatory responses (215). Since T cells are essential to immune responses, it is likely that differing expression of CCR5 and CXCR6 on these subsets in the different population groups may impact various disease outcomes depending on ethnicity.

B cells

While we saw a strong trend towards lower CCR5 density on B cells in black individuals compared to white individuals in this study, the 2012 study from our research group showed black individuals to have both a strongly significant higher frequency of CCR5-expressing B cells and significantly higher CCR5 density on B cells compared to white individuals (195). These contrasting results highlight the importance of validation cohorts, and investigation of CCR5 expression on B cells in a larger cohort of healthy individuals with different ethnic backgrounds would be beneficial to build on these findings.

Monocytes

In this study, black individuals had a significantly lower frequency of CCR5-expressing CD14^{+}CD16^{bright} monocytes compared to white individuals and while not significant, tended to have lower CCR5 expression on all monocytes subsets when compared with white individuals. Similarly, previous work from our research group found that, when excluding individuals with

CCR5Δ32, a strong trend towards a lower frequency of CCR5-expressing monocytes in black individuals compared to white individuals was found (195).

There was a strongly significant higher frequency of CXCR6-expressing $CD14^+CD16^+$ monocytes in black individuals compared to white individuals. $CD14^+CD16^+$ monocytes display inflammatory features and are recruited to sites of infection, where they then stimulate local and migrating immune cell antiviral function, promote inflammation, and differentiate into macrophages and dendritic cells (216,217). The inflammation and peripheral immune activation associated with persistent HIV-1 infection results in an increase of circulating $CD14^+CD16^+$ and $CD14^+CD16^{bright}$ monocytes (218,219), with the degree of CD16+ monocyte expansion being directly linked to the rate of disease progression (220). $CD14^+CD16^+$ monocytes stimulate antiviral Th1 immunity in infected tissue (217). When assessing ratios of CCR5 to CXCR6 expression on monocytes, more black individuals had a lower ratio of percentage of CCR5-expressing $CD14^+CD16^+$ and $CD14^+CD16^{bright}$ monocytes to CXCR6-expressing $CD14^+CD16^+$ and $CD14^+CD16^{bright}$ monocytes compared to white individuals. CCR5 expression did not correlate with CXCR6 expression (frequency and density) on any monocyte subset.

Monocytes and macrophages critically influence HIV transmission and viral spread occurring early in HIV-1 infection (220). In addition, chronically infected macrophages may provide a reservoir of inducible HIV-1 provirus (221). Therefore, differences in CCR5 and CXCR6 expression on these cells would likely have an effect on the ability of an individual to control HIV-1. Whether CXCR6 expression on monocytes contributes to the infectivity of monocytes, and the implications thereof on an HIV-1 reservoir, needs to be investigated further. It is important to determine whether the higher percentage of CXCR6-expressing $CD14^+CD16^+$ monocytes seen in black individuals compared to white individuals is protective in the context of HIV-1 infection through a preferential persistence of viraemia in CXCR6+ intermediate monocytes compared to CCR5+ monocytes, which may lead to a less pathogenic outcome, as seen in SIVsmm.

NK cells

The expression of CCR5 on NK cells has been found to play a significant role in both chemokinesis of NK cells in response to cytokines as well as the redistribution of NK cells to areas of active viral replication (222). Previous work from our research group showed significant population-specific

differences with regards to CCR5 expression on NK cells, with all except $CD56^{bright}$ NK cells having significantly different frequencies of CCR5 expressing cells (black individuals had significantly lower frequencies of CCR5-expressing $CD56^{+}$, $CD56^{dim}$ and $CD56^{+}CD16^{+}$ NK cells compared to white individuals) and all subsets, including total CD56+ NK cells, showing significant differences with regards to CCR5 density (black individuals had higher CCR5 density on all subsets compared to white individuals) (195).

Our study investigated CCR5 expression on these same subsets in addition to $CD56^{bright}CD16^{+}$, $CD56^{dim}CD16^{+}$, $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{-}$ NK cell subsets. Black individuals in our study had a significantly higher frequency of CCR5-expressing $CD56^{bright}CD16^{+}$ NK cells and a significantly lower frequency of CCR5-expressing $CD56^{dim}CD16^{-}$ NK cells compared to white individuals. Furthermore, we did not find a significant difference between populations with regards to CCR5 expression on total CD56+ NK cells. The results from this study therefore seem to differ slightly from the results seen in the 2012 study from our research group (195). With regards to CXCR6 expression, we did not see any significant differences with either CXCR6 frequency or CXCR6 density on any NK cell subset between population groups.

While CXCR6 expression (both frequency and density) was significantly higher on CD16- and $CD56^{bright}$ compared to CD16+ and $CD56^{dim}$ NK cell subsets, respectively, CCR5 expression (frequency and density) was higher on CD16- compared to CD16+ subsets but only the frequency of CCR5-expressing $CD56^{bright}$ and $CD56^{bright}CD16^{+}$ NK cells was higher than on the $CD56^{dim}$ counterparts. It has been shown that CCR5 and CXCR6, among other chemokine receptors, were expressed by higher percentages of $CD56^{bright}$ NK cells than $CD56^{dim}$ NK cells (223). Additionally, it was shown that while there was a higher percentage of CCR5-expressing $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells, CCR5 density did not differ between subsets (195).

The $CD56^{dim}CD16^{bright}$ NK cells represent at least 90% of all NK cells in peripheral blood and are therefore the major circulating subset (224). $CD56^{dim}CD16^{+}$ NK cells are more cytotoxic, while $CD56^{bright}CD16^{-}$ NK cells express the high-affinity interleukin-2 (IL-2) receptor CD25 and subsequently exhibit pronounced proliferation after exposure to IL-2 with a resulting production of greater amounts of cytokines (225). $CD56^{bright}$ NK cells predominate in lymph nodes and sites of inflammation (226).

Our results add to the knowledge that CCR5 and CXCR6 expression differs according to NK cell subset and depending on ethnicity. Within the $CD56^{bright}$, $CD56^{dim}$, CD16- and CD16+ NK cell populations, the difference in CCR5 and CXCR6 expression levels is likely to contribute to the differential trafficking capacity of these subsets, which likely influences their functions in both innate and adaptive immunity.

Immune activation and CCR5

Immune activation is a main driver of AIDS and non-AIDS linked morbidities in the course of HIV-1 disease, with increased T cell activation being an independent predictor of HIV-1 progression (190–192). In addition, T cell activation can be used to differentiate pathogenic and non-pathogenic SIV (155). There is a wide range of immune activation levels reported in individuals (227). CCR5 is not only a chemokine receptor but also a co-activation molecule expressed at the surface of T cells (190). The activation state of CD4+ cells, as measured by HLA-DR, has been shown to positively correlate with CCR5 expression (99,228). With CCR5 being upregulated on NK cells in individuals with HIV-1 viraemia, this indicates that CCR5 expression is modulated by the immune activation accompanying viral infection and inflammation (229).

In our study, the frequency of CCR5-expressing cells was higher among HLA-DR+ CD4+ and CD8+ T cell subsets, monocytes and NK cells compared to HLA-DR- subsets across both population groups, which is in agreement with what has been shown in other studies (228,230). When comparing populations, black individuals had significantly lower CCR5 density on HLA-DR+ CD4+ T cells, HLA-DR+ and HLA-DR- CD8+ T cells and HLA-DR- B cells, and lower CCR5 and CXCR6 density on HLA-DR+ monocytes compared to white individuals. Black individuals also had a significantly lower frequency of CCR5-expressing HLA-DR- monocytes compared to white individuals. What this may translate to with regards to HIV-1 control in black individuals compared to white individuals is uncertain, but in a study from our research group investigating CCR5 expression and immune quiescence in black South African HIV-1 controllers, CCR5 density on HLA-DR+ CD4+ and CD8+ T cells was significantly lower than in healthy controls (231), implying a protective effect of lower CCR5 density on these subsets and a potential increased likelihood of slower disease progression in black individuals compared to white individuals.

To assess the relationship between the frequency of HLA-DR-expressing CD4+ and CD8+ T cells and CCR5 expression in our population groups, correlation analyses were performed. Notably, populations differed considerably with regards to correlations of expression on CD4+ T cells and NK cells where black individuals tended to have negative correlations with HLA-DR+ CD4+ and CD8+ T cells and expression and white individuals tended to have positive correlations. The opposite was seen with regards to monocytes and CCR5 density. There were multiple significant associations on all cell types except B cells. Significant findings were mostly found with regards CCR5 density. When correlating the frequency of HLA-DR expressing CD4+ T cells with CCR5 expression, the majority of significant results were in the white population group, whereas when correlating the frequency of HLA-DR expressing CD8+ T cells with CCR5 expression, the majority of findings were in the black population group. Individuals of African descent have been shown to have increased immune activation (185,187,232–234).

Multiple monocyte and NK cell subsets had a significant association between frequency of HLA-DR-expressing CD4+ and CD8+ T cells and an increase in CCR5 density, most notably on the CD16+ subsets. Immune activation has been associated with an upregulation of CCR5 expression on NK cells (229).

Immune activation and CXCR6

The frequency of CXCR6-expressing cells was higher among HLA-DR+ CD4+ T cells compared to HLA-DR- subsets (white individuals only) and in HLA-DR+ monocytes and NK cells compared to HLA-DR- subsets across both population groups, while the frequency of CXCR6-expressing CD8+ T cells showed no difference between HLA-DR+ and HLA-DR- subsets. CXCR6 density did not differ between HLA-DR+ and HLA-DR- cells on any cell subset. The percentage of CXCR6-expressing B cells was lower among HLA-DR+ compared to HLA-DR- expressing cells for both population groups.

When investigating if activated HLA-DR+ CD4+ and CD8+ T cells correlated with CXCR6 expression, the black and white population groups tended to have different results on multiple cell types. While activated CD4+ T cells tended to similarly correlate with frequency of CXCR6 expression on monocytes across population groups, when it came to CXCR6 density, in white individuals, activated CD4+ cells significantly negatively correlated with expression on

$CD14^{+}CD16^{bright}$ monocytes and showed a general pattern of negative correlation with CXCR6 expression, whereas activated $CD4^{+}$ T cells tended to positively correlate with CXCR6 density on monocytes in black individuals. Activated CD4+ and CD8+ T cells tended to negatively correlate with CXCR6 expression on NK cells in black individuals and positively correlate with CXCR6 expression on NK cells in white individuals.

Activated CD8+ T cells positively correlated with frequency of CXCR6 expression across both population groups with multiple significant correlations, primarily in the $CD56^{dim}$ and $CD16^{+}$ subsets. It has been shown that $CD56^{dim}CD16^{+}$ NK cells exhibit early and rapid IFN-γ production and cytolytic activity, necessary during the early phases of innate responses (235). The main findings seen are with regards to CD8+ T cells, monocytes (specifically CD16+ monocytes) and NK cells (primarily $CD56^{dim}$ and CD16+ subsets), all cell types implicated in the Th1 and inflammation response. Since CXCR6 is associated with the recruitment of cells to areas of inflammation (236,237), it is perhaps understandable that CXCR6 expression on these inflammatory cell types correlates with activated T cells.

The percentage of CXCR6-expressing terminally differentiated CD8+ T cells and the percentage of CXCR6-expressing NK subsets positively correlated with activated CD8+ T cells in both the black and white populations, as well as in the combined group. The presence of increased terminally differentiated T cells during HIV-1 infection are often suggestive of exhaustion, with a resulting dysregulation of T cell homeostasis and function and a subsequent rapid progression of HIV disease (238). It has been shown that CXCR6-expressing NK cells upregulate TNF-related apoptosis-inducing ligand (TRAIL) (133). TRAIL has been found to be involved in the pathogenesis of HIV-1 by causing CD4+ T cell death and was shown to be higher in the plasma of people living with HIV-1 compared with uninfected individuals. Additionally, patients on ART had decreased TRAIL levels in their plasma that correlated with a reduction in viral load (239). CXCR6-expressing NK cells activated by antigenic exposure are targets of HIV-1 and are depleted by R5-tropic HIV (125). NK cell activation is increased in people living with HIV-1 who progress to AIDS, but not in elite and viraemic controllers (240). Hyperactivation of T cells, predominantly of CD8+ T cells, is a hallmark of chronic HIV-1 infection (241).

A study on black South Africans from Soweto found a general homoegeneity of the group, with the average level of admixture not hypothesised to significantly affect the outcome of disease

association studies (242). White individuals in our study, while having a South African nationality, may have varying ethnic backgrounds contributing to a potential genetic diversity and lack of homogeneity in this group of controls, which is something to consider. Additionally, while age- and sex-matched, sociodemographic factors may differ between groups. For example, previous work from our research group (Shalekoff, unpublished data), demonstrated that significantly more black South Africans were seropositive for human cytomegalovirus (HCMV) compared to white individuals. While it is unclear whether the overburden of HCMV in non-white population groups is due to socioeconomic factors or host genetics, this may have implications for our study findings, since cellular activation level differences could be attributed to the effect of sociodemographic factors versus ethnicity alone.

This study gives novel insight into constitutive CCR5 and CXCR6 expression levels on CD4+ and CD8+ T cells, B cells, monocytes and NK cells in HIV-1-uninfected black and white South Africans. While CCR5 and CXCR6 act as coreceptors for HIV-1 and SIV, they are also both important molecules with regards to chemotaxis and are involved in both immune and inflammatory responses. It is important to assess differential CCR5 and CXCR6 expression on multiple cell types in healthy individuals without the confounding effect of HIV-1 infection. There were significant population-specific differences in expression levels of both CCR5 and CXCR6 and multiple associations with cell activation and CCR5 and CXCR6 expression. We also found that CCR5 and CXCR6 expression significantly correlated on multiple cell subsets. A deeper understanding of the causes and implications of the variation seen in CCR5 and CXCR6 expression between population groups will help to further gain understanding into the role of CCR5 and CXCR6, alone and in combination, in HIV-1 control, as well as in other diseases.

CHAPTER FIVE

5. Influence of sex and age on CCR5 and CXCR6 expression in two ethnically distinct South African population groups

5.1 Introduction

As outlined in Chapter 4, the CCR5 and CXCR6 chemokines are involved in both the acquisition and progression of HIV-1, HIV-2 and SIV. A variety of factors can affect the expression levels of chemokines and their associated receptors, including sex and age. Multiple publications have posited why sex should be considered in pre-clinical, clinical, and population research (243,244). There are sex differences with regards to symptoms as well as clinical presentations of illness, the reliability of diagnostic tests, and in the response to treatments (245). There is also sex bias at the level of epigenetic marking and gene expression (246). Multiple studies have shown an effect of sex on expression levels of chemokines and cytokines on various cell types in various pathologies (247–249) and estrogen has been shown to increase CCR1 and CCR5 expression and decrease tumour-necrosis factor (TNF) production by CD4+ T cells (250).

Sex differences also impact HIV-1 disease progression (251–253). Females living with HIV-1, in general, have higher levels of innate and adaptive immune responses than males (254). In SIV-infected macaques, central memory CD4+ T cells from females differentially regulate a significantly larger number of genes at day 4 post-infection when compared to males, with female macaques having significantly higher levels of the CCL2, interferon–inducible T cell alpha chemoattractant (I-TAC) and macrophage migration inhibitory factor (MIF) cytokines, suggesting that early innate cytokine responses to SIV is skewed towards a more pro-inflammatory phenotype in female macaques (255). In general, a larger proportion of HIV-1 controllers are female (256–258). In a small cardiac study, women were found to have lower CCR5 expression in peripheral blood than men (259). Furthermore, the percentage of CCR5+ CD4+ T cells has been found to be lower in untreated women living with HIV-1 compared to men, while the density of CCR5 on CD4+ T cells was lower in HIV-1 uninfected women compared to men (260,261).

Using animal models, expression studies showed that aging could be associated with the expression of up to 75% of genes (262). In mice thymocytes, age-associated changes in the expression of genes involved in T cell receptor signaling, antigen presentation and lymphocyte development and

function have been identified, including an increased expression of CXCR4 and CXCR6 with age, and a decrease in CCL25 (263). Aging has been associated with an increased CCR5 expression on T cells in mice and in humans (264,265) and CCR5 expression was found to be significantly lower in cord blood from children compared to adults (266–268).

Given the documented differences between sexes and the impact of age on expression levels, the relationship between sex and age with regards to CCR5 and CXCR6 expression in two HIV-1 uninfected ethnic population groups in South Africa was investigated. Background, constitutive CCR5 and CXCR6 expression on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells from both black and white healthy HIV-1-uninfected South Africans was assessed and it was determined if sex and age affected expression levels.

5.2 Materials and Methods

5.2.1 Sample population

Refer to Chapter 2, Materials and Methods section 2.1. Briefly, healthy HIV-1-uninfected South African black (n=17) and white (n=21) individuals were recruited and were age- and sex-matched.

5.2.2 Flow Cytometry

CCR5 and CXCR6 expression on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells for each individual were determined as described in Chapter 2, Materials and Methods section 2.7 and assessed as outlined in Materials and Methods section 2.9.

5.2.3 Statistical analyses

Statistical analyses were performed as described in Chapter 2, Materials and Methods section 2.10.

5.3 Results

5.3.1 Sex differences

The influence of sex on CCR5 and CXCR6 expression on all cell subsets was investigated and a summary of significant findings can be seen in Figure 5.1. Of the few significant results, p values were not high enough to survive correction for multiple comparisons. Tables 5.1 – 5.4 show all results.

5.3.1.1 CCR5 expression and sex

Results noticeably varied depending on the population group studied. The only finding in the black population group was a higher percentage of CCR5-expressing central memory CD8+ T cells in males compared to females, which was replicated in the total group findings (p=0.008 and p=0.015, respectively; Table 5.1). Although not significant in white South Africans, males still had a higher median percentage of CCR5-expressing central memory CD8+ T cells (35.7%) compared to females (23.5%). In the total group, the percentage of CCR5-expressing terminally differentiated CD8+ T cells was higher in females compared to males (p=0.019; Table 5.1). While this finding did not reach significance in the individual population groups, there was a trend towards a higher percentage of CCR5-expressing terminally differentiated CD8+ T cells in females compared to males in black and white individuals (p=0.093 and p=0.072, respectively; Table 5.1). Males had a higher percentage of CCR5-expressing $CD56^{bright}CD16^{-}$ NK cells compared to females in the white population group (p=0.016; Table 5.1). CCR5 density did not differ on any cell subset with regards to sex (Table 5.2).

5.3.1.2 CXCR6 expression and sex

All significant findings assessing age and CXCR6 expression were seen in the white population group. In white individuals, males had a significantly increased percentage of CXCR6-expressing B cells (p=0.036; Table 5.3), naïve (p=0.024; Table 5.3), transitional memory (p=0.043; Table 5.3) and effector memory (p=0.029; Table 5.3) CD4+ T cells compared to females, and an increased CXCR6 density on terminally differentiated CD4+ T cells (p=0.036; Table 5.4). Females had a significantly increased percentage of CXCR6-expressing transitional memory CD8+ T cells compared to males (p=0.029).

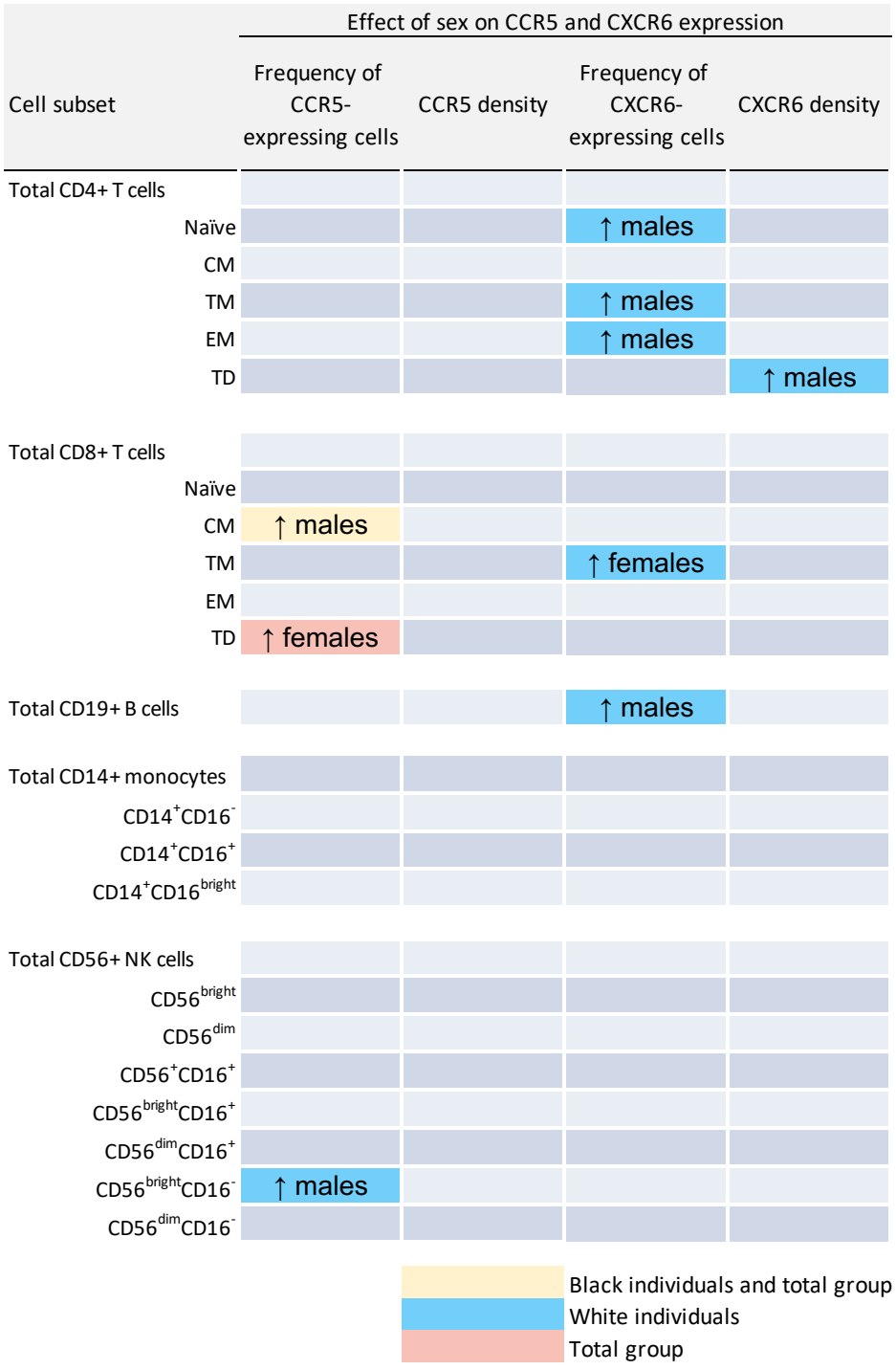


Figure 5.1 - Summary of significant sex effects on CCR5 and CXCR6 expression on total CD4+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD4+ T cell subsets, total CD8+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets, CD19+ B cells, total CD14+ monocytes and monocyte subsets and total CD56+ NK cell subsets.

Table 5.1 - Percentage of CCR5-expressing cells differences by sex.

Median percentage of CCR5-expressing cells (IQR)									
	Black South Africans			White South Africans			Total Group		
Cell subset	Female (n=9)	Male (n=8)	P value	Female (n=11)	Male (n=10)	P value	Female (n=20)	Male (n=18)	P value
CD4+ T cells									
Total CD4+	13.4 (11.9-18.2)	14.7 (13.0-15.9)	0.963	12.6 (9.8-14.2)	12.6 (11.6-15.7)	0.557	12.7 (10.0-16.5)	13.7 (11.8-16.0)	0.553
Naïve	0.4 (0.3-0.6)	0.5 (0.4-0.8)	0.370	0.5 (0.4-0.5)	0.5 (0.4-0.7)	0.152	0.5 (0.4-0.6)	0.5 (0.4-0.8)	0.112
CM	7.6 (6.8-11.1)	8.8 (7.5-11.9)	0.743	7.6 (6.8-10.2)	8.8 (8.2-11.9)	0.387	7.6 (6.7-11.3)	8.8 (8.0-12.0)	0.331
TM	38.8 (25.9-44.9)	43.8 (32.8-48.4)	0.541	37.2 (35.0-44.5)	38.6 (28.0-45.4)	0.705	38.8 (33.4-45.9)	41.0 (31.9-46.8)	0.874
EM	62.4 (40.1-66.8)	62.2 (54.4-68.4)	0.481	60.6 (50.9-68.6)	64.7 (51.1-68.9)	0.809	61.5 (47.0-68.4)	62.9 (52.6-68.9)	0.460
TD	40.0 (25.9-46.5)	26.7 (10.5-36.1)	0.277	37.8 (25.1-51.1)	31.9 (25.1-34.8)	0.387	38.9 (24.8-50.9)	30.8 (21.1-34.9)	0.141
CD8+ T cells									
Total CD8+	27.6 (24.9-39.9)	27.5 (19.7-33.1)	0.815	32.0 (28.5-40.7)	32.4 (24.8-35.5)	0.705	31.8 (25.4-41.6)	30.0 (22.1-35.5)	0.393
Naïve	0.7 (0.6-1.0)	0.8 (0.6-1.4)	0.606	1.0 (0.8-1.1)	1.1 (0.9-1.7)	0.223	0.8 (0.6-1.1)	1.0 (0.8-1.6)	0.196
CM	26.0 (24.4-30.2)	**37.2 (31.6-44.7)**	**0.008**	23.5 (22.7-25.1)	35.7 (27.8-39.3)	0.251	25.7 (22.7-33.1)	**36.4 (31.4-43.4)**	**0.015**
TM	74.7 (66.1-84.0)	73.7 (72.1-76.5)	0.815	72.4 (69.4-80.0)	60.2 (47.7-66.6)	0.061	74.5 (66.8-81.3)	69.8 (60.2-75.7)	0.119
EM	74.0 (49.3-83.4)	69.7 (56.1-76.6)	0.541	81.6 (70.8-86.9)	68.2 (54.3-78.7)	0.099	79.5 (61.8-86.7)	69.7 (54.0-77.9)	0.082
TD	52.3 (32.9=60.1)	27.3 (21.1-46.7)	0.093	44.6 (41.8-61.7)	35.9 (26.0-46.0)	0.072	**45.6 (36.6-60.9)**	32.9 (23.3-47.7)	**0.019**
CD19+ B cells									
Total CD19+	1.2 (1.0-1.5)	2.0 (1.1-2.6)	0.606	1.2 (1.0-1.3)	1.3 (0.9-1.6)	0.468	1.2 (1.0-1.3)	1.4 (1.0-2.2)	0.534
CD14+ monocytes									
Total CD14+	2.5 (1.7-4.6)	3.6 (3.0-4.2)	0.200	4.9 (2.8-6.6)	4.4 (2.0-4.8)	0.251	3.7 (1.9-6.1)	3.8 (2.5-4.8)	0.851
$CD14^{+}CD16^{-}$	2.2 (1.7-4.4)	2.9 (2.4-4.4)	0.481	4.4 (3.7-5.3)	3.7 (1.9-4.3)	0.132	4.1 (2.0-5.3)	3.2 (2.3-4.4)	0.409
$CD14^{+}CD16^{+}$	8.4 (4.2-12.7)	8.6 (6.1-11.6)	0.888	13.4 (8.4-16.7)	9.4 (5.5-13.0)	0.197	12.0 (7.0-16.6)	8.6 (5.8-11.9)	0.346
$CD14^{dim}CD16^{bright}$	10.3 (3.5-17.1)	9.9 (8.1-13.1)	0.888	18.6 (14.7-23.3)	12.3 (10.0-18.6)	0.132	17.0 (9.9-20.1)	11.7 (8.5-14.6)	0.377
CD56+ NK cells									
Total CD56+	12.4 (6.4-21.0)	13.4 (7.2-18.1)	0.815	11.2 (8.0-14.9)	11.1 (8.9-14.2)	0.918	11.8 (7.3-18.0)	11.1 (8.2-17.1)	0.828
$CD56^{bright}$	33.8 (11.8-36.1)	25.8 (14.8-37.1)	0.963	20.6 (17.7-22.8)	19.9 (14.7-21.5)	0.654	21.1 (16.5-32.8)	20.3 (13.9-32.7)	0.988
$CD56^{dim}$	10.8 (6.5-20.2)	11.0 (5.0-17.7)	0.606	10.4 (6.2-13.6)	10.4 (7.9-12.2)	0.809	10.6 (6.3-15.2)	10.4 (6.6-14.9)	0.897
$CD56^{+}CD16^{+}$	6.7 (5.0-19.3)	9.2 (3.4-16.1)	0.606	6.8 (2.4-7.2)	5.8 (4.1-6.6)	0.918	6.8 (3.4-8.7)	5.8 (3.6-10.7)	0.828
$CD56^{bright}CD16^{+}$	14.4 (6.9-26.6)	12.2 (7.5-27.7)	0.743	5.8 (5.0-12.1)	7.8 (4.9-12.3)	0.863	7.1 (5.2-15.1)	8.0 (6.1-17.1)	0.654
$CD56^{dim}CD16^{+}$	7.9 (5.8-24.2)	10.4 (3.8-17.8)	0.673	6.5 (2.6-7.5)	5.0 (3.3-6.5)	0.918	6.8 (3.1-9.6)	5.0 (3.3-9.2)	0.654
$CD56^{bright}CD16^{-}$	22.9 (13.6-42.6)	26.3 (20.8-42.9)	0.481	23.9 (21.0-32.4)	**38.8 (33.8-41.5)**	**0.016**	23.4 (13.6-33.8)	36.5 (23.5-41.5)	0.067
$CD56^{dim}CD16^{-}$	19.2 (12.7-28.0)	24.3 (17.0-29.4)	0.481	27.2 (23.7-36.0)	36.5 (27.8-46.1)	0.251	26.3 (17.7-30.4)	28.7 (22.4-38.4)	0.217

Where significant differences are observed ($p<0.05$), the highest value in the comparison is bolded and shaded.

Table 5.2 - CCR5 density differences by sex.

Median number of CCR5 molecules (IQR)									
	Black South Africans			White South Africans			Total Group		
Cell subset	Female (n=9)	Male (n=8)	P value	Female (n=11)	Male (n=10)	P value	Female (n=20)	Male (n=18)	P value
CD4+ T cells									
Total CD4+	681 (564-875)	720 (651-756)	0.888	758 (654-846)	805 (686-887)	0.705	737 (590-869)	762 (652-807	0.806
Naïve	373 (274-403)	307 (240-381)	0.815	405 (344-454)	396 (351-446)	0.863	382 (311-415)	363 (282-410)	0.740
CM	668 (446-777)	635 (546-688)	0.888	711 (687-846)	789 (676-836)	0.918	704 (651-826)	694 (591-812)	0.806
TM	873 (756-1045)	786 (713-863)	0.277	868 (784-1035)	838 (735-1063)	0.973	870 (769-1046)	824 (714-968)	0.377
EM	1025 (793-1115)	844 (719-925)	0.236	837 (807-1089)	914 (769-1099)	0.973	944 (792-1130)	889 (745-1004)	0.409
TD	731 (463-897)	546 (526-632)	0.481	748 (668-942)	648 (579-917)	0.251	740 (611-907)	590 (532-834)	0.133
CD8+ T cells									
Total CD8+	1017 (846-1258)	971 (883-1053)	0.963	1369 (1231-1484)	1219 (939-1441)	0.468	1256 (998-1388)	1034 (897-1257)	0.303
Naïve	422 (299-439)	371 (330-479)	0.963	388 (371-407)	433 (328-538)	0.863	389 (353-423)	406 (328-538)	0.553
CM	929 (630-1122)	849 (750-938)	0.673	1106 (962-1236)	980 (778-1096)	0.468	1058 (840-1179)	930 (737-1060)	0.276
TM	1150 (888-1463)	1065 (1033-1225)	0.743	1469 (1298-1724)	1168 (829-1439)	0.085	1435 (1097-1550)	1065 (931-1411)	0.099
EM	1138 (988-1579)	1160 (1098-1363)	0.888	1540 (1405-1695)	1511 (926-1731)	0.705	1508 (1110-1654)	1270 (1006-1568)	0.534
TD	684 (545-1006)	590 (552-777)	0.370	740 (683-889)	714 (632-759)	0.387	737 (649-964)	672 (573-762)	0.251
CD19+ B cells									
Total CD19+	573 (463-633)	589 (437-662)	0.888	583 (508-763)	652 (478-875)	0.605	578 (465-637)	645 (449-748)	0.534
CD14+ monocytes									
Total CD14+	596 (553-895)	592 (570-701)	0.673	726 (601-893)	636 (574-733)	0.314	666 (577-908)	601 (572-727)	0.346
$CD14^{+}CD16^{-}$	539 (505-717)	613 (557-677)	0.423	615 (555-779)	589 (531-661)	0.468	594 (533-755)	607 (543-667)	0.942
$CD14^{+}CD16^{+}$	603 (538-992)	743 (717-769)	0.888	792 (705-916)	795 (639-820)	0.557	784 (596-979)	761 (679-808)	0.593
$CD14^{dim}CD16^{bright}$	495 (474-791)	616 (530-777)	0.743	759 (623-985)	631 (585-850)	0.282	747 (495-970)	622 (554-820)	0.633
CD56+ NK cells									
Total CD56+	365 (296-453)	347 (306-399)	0.815	392 (337-468)	413 (381-507)	0.468	378 (318-457)	384 (329-459)	0.675
$CD56^{bright}$	338 (312-485)	361 (314-413)	0.673	354 (330-440)	416 (369-533)	0.468	353 (323-463)	399 (323-498)	0.784
$CD56^{dim}$	370 (283-402)	366 (307-373)	0.963	402 (347-423)	428 (342-479)	0.863	377 (333-418)	369 (325-470)	0.897
$CD56^{+}CD16^{+}$	334 (262-390)	297 (278-377)	0.963	363 (258-374)	344 (297-389)	0.605	349 (262-381)	328 (290-389)	0.718
$CD56^{bright}CD16^{+}$	305 (261-359)	331 (241-369)	1.000	369 (327-417)	333 (281-403)	0.349	342 (299-408)	333 (254-383)	0.478
$CD56^{dim}CD16^{+}$	302 (285-329)	299 (242-347)	0.963	333 (254-349)	335 (289-375)	0.468	313 (260-338)	324 (268-363)	0.460
$CD56^{bright}CD16^{-}$	369 (346-624)	502 (335-579)	0.815	405 (369-548)	476 (348-499)	0.809	396 (354-596)	476 (323-544)	0.675
$CD56^{dim}CD16^{-}$	530 (398-573)	417 (402-498)	0.541	448 (409-523)	487 (370-526)	0.512	487 (407-573)	453 (384-526)	0.264

Table 5.3 - Percentage of CXCR6-expressing cells differences by sex.

Median percentage CXCR6 expressing cells (IQR)									
	Black South Africans			White South Africans			Total Group		
Cell subset	Female (n=9)	Male (n=8)	P value	Female (n=11)	Male (n=10)	P value	Female (n=20)	Male (n=18)	P value
CD4+ T cells									
Total CD4+	1.9 (1.3-2.2)	1.9 (1.6-2.9)	0.963	1.5 (1.2-1.8)	2.5 (2.0-3.0)	0.061	1.6 (1.2-2.0)	2.2	0.105
Naïve	0.4 (0.4-0.6)	0.3 (0.2-0.3)	0.074	0.2 (0.2-0.3)	**0.4 (0.4-0.5)**	**0.024**	0.3 (0.2-0.5)	0.4	0.613
CM	1.3 (1.0-1.4)	1.0 (1.0-1.2)	0.541	1.2 (1.0-1.4)	1.7 (1.3-2.0)	0.099	1.2 (1.0-1.4)	1.3	0.478
TM	9.0 (6.2-11.3)	8.8 (8.4-9.1)	0.963	6.9 (5.7-8.3)	**9.0 (8.0-10.5)**	**0.043**	8.0 (5.8-9.1)	8.8	0.072
EM	15.7 (9.9-19.2)	15.0 (10.6-21.3)	0.963	13.0 (8.6-14.3)	**17.3 (14.1-23.7)**	**0.029**	13.2 (8.8-15.8)	16.6	0.141
TD	5.3 (3.3-10.0)	5.5 (1.4-9.1)	0.370	4.3 (3.5-8.7)	6.1 (5.0-9.9)	0.557	4.8 (3.3-10.1)	6.1	0.874
CD8+ T cells									
Total CD8+	7.0 (3.3-12.1)	8.0 (4.4-10.6)	1.000	12.4 (8.6-16.9)	10.2 (5.8-12.2)	0.152	10.6 (7.0-15.8)	8.9	0.377
Naïve	0.4 (0.3-0.7)	0.4 (0.3-0.7)	0.963	0.7 (0.5-0.8)	0.8 (0.6-0.9)	0.314	0.6 (0.4-0.8)	0.7	0.460
CM	4.4 (2.8-5.6)	4.0 (2.5-5.3)	0.673	2.9 (2.3-3.6)	3.3 (2.6-3.7)	0.468	3.3 (2.3-4.7)	3.3	0.784
TM	24.8 (13.4-31.9)	20.0 (14.8-35.3)	0.888	**25.0 (19.7-45.1)**	19.0 (12.0-20.7)	**0.029**	25 (18.2-41.1)	19.0	0.059
EM	24.1 (15.8-53.1)	30.0 (13.3-38.1)	0.888	47.0 (33.6-70.1)	40.5 (25.9-54.3)	0.314	36.9 (22.9-59.5)	32.4	0.409
TD	14.9 (3.1-24.2)	6.2 (3.6-11.7)	0.277	6.6 (5.6-13.5)	6.6 (3.1-9.8)	0.282	10.5 (4.9-16.9)	6.6	0.087
CD19+ B cells									
Total CD19+	0.7 (0.6-1.2)	0.5 (0.4-0.8)	0.236	0.4 (0.4-0.7)	**0.9 (0.8-1.0)**	**0.036**	0.6 (0.4-0.8)	0.8	0.346
CD14+ monocytes									
Total CD14+	0.4 (0.3-0.4)	0.5 (0.5-0.6)	0.074	0.5 (0.3-0.5)	0.5 (0.4-0.6)	0.512	0.4 (0.3-0.5)	0.5	0.082
$CD14^{+}CD16^{-}$	0.5 (0.4-0.5)	0.5 (0.4-0.6)	0.815	0.4 (0.3-0.5)	0.5 (0.5-0.6)	0.114	0.4 (0.4-0.5)	0.5	0.196
$CD14^{+}CD16^{+}$	1.8 (1.4-2.0)	1.5 (1.3-2.3)	0.888	1.0 (0.9-1.4)	1.1 (0.7-1.3)	0.605	1.4 (1.0-1.9)	1.3	0.806
$CD14^{dim}CD16^{bright}$	1.5 (1.2-1.7)	1.2 (0.8-2.0)	0.673	1.0 (0.7-1.9)	0.9 (0.7-2.4)	0.863	1.3 (0.8-1.7)	1.0	1.000
CD56+ NK cells									
Total CD56+	3.4 (2.3-6.4)	3.6 (2.4-4.5)	0.815	4.3 (3.5-5.0)	4.0 (3.3-5.4)	0.809	3.8 (2.8-5.6)	4.0	0.762
$CD56^{bright}$	9.1 (4.0-14.2)	10.2 (9.3-14.6)	0.276	14.4 (9.5-15.5)	10.9 (10.2-12.6)	0.756	11.9 (8.7-15.5)	10.8	0.613
$CD56^{dim}$	3.4 (2.6-4.5)	2.9 (2.0-3.5)	0.481	3.4 (2.6-4.0)	3.2 (2.8-4.6)	0.809	3.4 (2.6-4.4)	3.1	0.654
$CD56^{+}CD16^{+}$	2.7 (1.9-4.0)	2.4 (1.6-3.0)	0.541	2.3 (1.8-3.1)	2.1 (1.9-3.9)	0.809	2.5 (1.9-3.3)	2.1	0.718
$CD56^{bright}CD16^{+}$	3.6 (1.8-7.6)	4.8 (3.6-6.1)	0.815	5.3 (4.5-6.7)	5.5 (4.8-7.2)	0.605	5.1 (3.6-7.1)	5.4	0.696
$CD56^{dim}CD16^{+}$	2.2 (1.7-3.6)	2.2 (1.7-2.6)	0.606	2.0 (1.7-3.2)	2.3 (2.0-3.8)	0.387	2.1 (1.7-3.3)	2.3	0.851
$CD56^{bright}CD16^{-}$	11.1 (7.3-19.9)	22.8 (15.6-30.5)	0.200	18.7 (15.3-22.9)	17.9 (14.8-22.5)	0.863	17.2 (10.8-22.8)	19.0	0.264
$CD56^{dim}CD16^{-}$	7.1 (4.6-10.0)	6.6 (4.5-9.0)	0.673	6.7 (5.1-8.7)	6.4 (5.0-9.7)	0.973	7.0 (4.7-9.5)	6.5	0.828

Where significant differences are observed ($p<0.05$), the highest value in the comparison is bolded and shaded.

Table 5.4 - CXCR6 density differences by sex.

Cell subset	Median number of CXCR6 molecules (IQR)								
	Black South Africans			White South Africans			Total Group		
	Female (n=9)	Male (n=8)	P value	Female (n=11)	Male (n=10)	P value	Female (n=20)	Male (n=18)	P value
CD4+ T cells									
Total CD4+	976 (831-1156)	819 (705-1052)	0.370	859 (808-900)	912 (857-953)	0.282	874 (818-1009)	893	0.942
Naïve	467 (429-501)	427 (347-612)	0.888	482 (443-540)	493 (474-596)	0.282	474 (438-516)	484	0.534
CM	672 (561-692)	609 (554-699)	0.815	667 (576-727)	710 (636-810)	0.282	670 (572-721)	676	0.573
TM	875 (745-1149)	695 (595-901)	0.200	720 (637-791)	761 (732-796)	0.756	763 (666-936)	759	0.496
EM	729 (646-990)	742 (684-858)	0.888	698 (617-811)	865 (754-893)	0.251	713 (630-874)	771	0.426
TD	561 (506-991)	488 (270-956)	0.321	587 (445-794)	**982 (855-1336)**	**0.036**	581 (484-888)	925	0.377
CD8+ T cells									
Total CD8+	944 (770-1071)	680 (574-1042)	0.236	815 (688-856)	857 (690-895)	0.557	829 (725-1021)	796	0.553
Naïve	504 (448-531)	429 (359-539)	0.481	459 (360-481)	473 (450-536)	0.314	462 (401-524)	460	0.806
CM	633 (570-753)	557 (496-705)	0.321	662 (535-859)	643 (579-786)	0.973	633 (557-831)	598	0.613
TM	794 (719-1010)	698 (471-806)	0.200	719 (626-792)	701 (588-817)	0.863	767 (645-829)	701	0.426
EM	785 (677-954)	776 (554-885)	0.743	739 (712-843)	725 (573-803)	0.605	741 (687-854)	760	0.573
TD	521 (514-759)	497 (404-527)	0.200	519 (448-531)	454 (367-485)	0.197	519 (477-550)	477	0.063
CD19+ B cells									
Total CD19+	444 (399-457)	428 (385-460)	0.606	470 (438-495)	506 (413-525)	0.756	456 (424-481)	450	0.874
CD14+ monocytes									
Total CD14+	1335 (1216-1679)	1529 (1171-1688)	0.963	1814 (1450-2081)	1491 (1369-1684)	0.223	1624 (1261-1955)	1502	0.393
$CD14^{+}CD16^{-}$	1123 (1038-1287)	1364 (1133-1478)	0.321	1428 (1130-2112)	1349 (1181-1477)	0.512	1235 (1064-1574)	1352	0.851
$CD14^{+}CD16^{+}$	1153 (1116-1922)	1259 (1104-1396)	0.606	1802 (1157-2254)	1535 (1320-1739)	0.654	1529 (1116-2185)	1327	0.593
$CD14^{dim}CD16^{bright}$	1140 (1081-1419)	1216 (725-1598)	0.743	1407 (1026-1690)	1231 (1046-1777)	0.863	1278 (1052-1617)	1231	0.696
CD56+ NK cells									
Total CD56+	762 (708-961)	641 (637-739)	0.321	846 (705-885)	848 (814-877)	0.973	804 (705-942)	814	0.393
$CD56^{bright}$	1242 (910-1331)	925 (838-1024)	0.093	927 (893-1034)	1010 (956-1119)	0.426	985 (901-1263)	989	0.426
$CD56^{dim}$	760 (638-852)	572 (538-665)	0.277	664 (585-740)	700 (649-773)	0.605	676 (599-798)	661	0.460
$CD56^{+}CD16^{+}$	740 (560-803)	591 (515-632)	0.321	635 (570-716)	668 (611-709)	0.557	650 (554-770)	631	0.654
$CD56^{bright}CD16^{+}$	866 (647-1074)	619 (490-757)	0.114	708 (661-813)	620 (610-850)	0.654	732 (651-901)	629	0.141
$CD56^{dim}CD16^{+}$	725 (564-814)	549 (483-621)	0.321	588 (566-654)	612 (580-680)	0.557	603 (563-732)	599	0.573
$CD56^{bright}CD16^{-}$	1040 (875-1281)	1015 (851-1191)	0.673	1029 (964-1170)	1199 (950-1284)	0.282	1034 (915-1222)	1135	0.675
$CD56^{dim}CD16^{-}$	918 (669-1083)	757 (725-908)	0.815	864 (798-943)	857 (771-1039)	0.809	892 (770-1030)	812	0.675

Where significant differences are observed ($p<0.05$), the highest value in the comparison is bolded and shaded.

5.3.2 Age effects

The age range of participants in our study was broad, ranging from 25 to 63 years of age, allowing us to investigate the relationship between CCR5 and CXCR6 expression and age on all cell subsets studied (Figure 5.2).

5.3.2.1 CCR5 expression and age

Older age associated with a significantly lower frequency of CCR5-expressing CD4+ T cells in black individuals (R=-0.522, p=0.032) and in the total group (R=-0.355, p=0.029). All other significant associations were seen in the white population group only and, except for older age associating with a higher percentage of CCR5-expressing CD8+ T cells (R=0.518, p=0.016), older age associated with lower CCR5 expression for all significant findings. Older age strongly associated with a lower percentage of CCR5-expressing total CD56+ NK cells (R=-0.583, p=0.006), $CD56^{dim}$ NK cells (R=-0.600, p=0.004), $CD56^{+}CD16^{+}$ NK cells (-0.639, p=0.002), $CD56^{bright}CD16^{+}$ NK cells (R=-0.561, p=0.008) and $CD56^{dim}CD16^{+}$ NK cells (R=-0.607, p=0.004), and a lower CCR5 density on $CD56^{bright}$ NK cells (R=-0.479, p=0.028) and $CD56^{dim}CD16^{+}$ NK cells (R=-0.522, p=0.015).

5.3.2.2 CXCR6 expression and age

Again, significant findings were primarily seen in the context of white individuals. Older age associated with a significantly lower frequency of CXCR6-expressing naïve CD4+ T cells (R=-0.494, p=0.023), $CD56^{+}CD16^{+}$ NK cells (R=-0.589, p=0.005) and $CD56^{dim}CD16^{+}$ NK cells (R=-0.668, p=0.0009), and associated with a higher percentage of CXCR6-expressing naïve CD8+ T cells (R=0.500, p=0.021). In the total group, older age associated with a significantly lower frequency of CXCR6-expressing total CD4+ T cells (R=-0.323, p=0.048), effector memory CD4+ T cells (R=-0.355, p=0.029) and $CD56^{dim}CD16^{+}$ NK cells (R=-0.336, p=0.039), and associated with a higher percentage of CXCR6-expressing naïve CD8+ T cells (R=0.487, p=0.002).

With regards to CXCR6 density, all significant findings associated older age with a decreased density (Figure 5.2). The only finding in black individuals was on effector memory CD4+ T cells (R=-0.518, p=0.033). In white individuals, older age associated with lower CXCR6 density on naïve CD8+ T cells (R=-0.495, p=0.022), central memory CD8+ T cells (R=-0.533, p=0.013), $CD56^{+}CD16^{+}$ NK cells (R=-0.471, p=0.031), $CD56^{bright}CD16^{+}$ NK cells (R=-0.469, p=0.032) and

$CD56^{dim}CD16^{+}$ NK cells (R=-0.453, p=0.039). Total group findings were on total CD8+ T cells (R=-0.366, p=0.024), naïve CD8+ T cells (R=-0.437, p=0.006) and central memory CD8+ T cells (R=-0.387, p=0.016). Interestingly, while age associated with a significantly higher percentage of CXCR6-expressing naïve CD8+ T cells, age associated with a significantly lower CXCR6 density on these same cells.

5.3.2.3 Comparing CCR5 and CXCR6 findings

Upon visual inspection of the heatmap, it is interesting to note some striking differences between CCR5 and CXCR6 findings. Whereas age tended to associate with a higher percentage of CCR5-expressing monocytes and a higher CCR5 density on monocytes across population groups, age associated with a lower percentage of CXCR6-expressing monocytes and a lower CXCR6 density on monocytes. On CD8+ T cells, age tended to associate with a higher CCR5 density but a lower CXCR6 density. A consistent finding was age strongly associating with a lower CCR5 and CXCR6 expression (both frequency and density) on NK cell subsets.

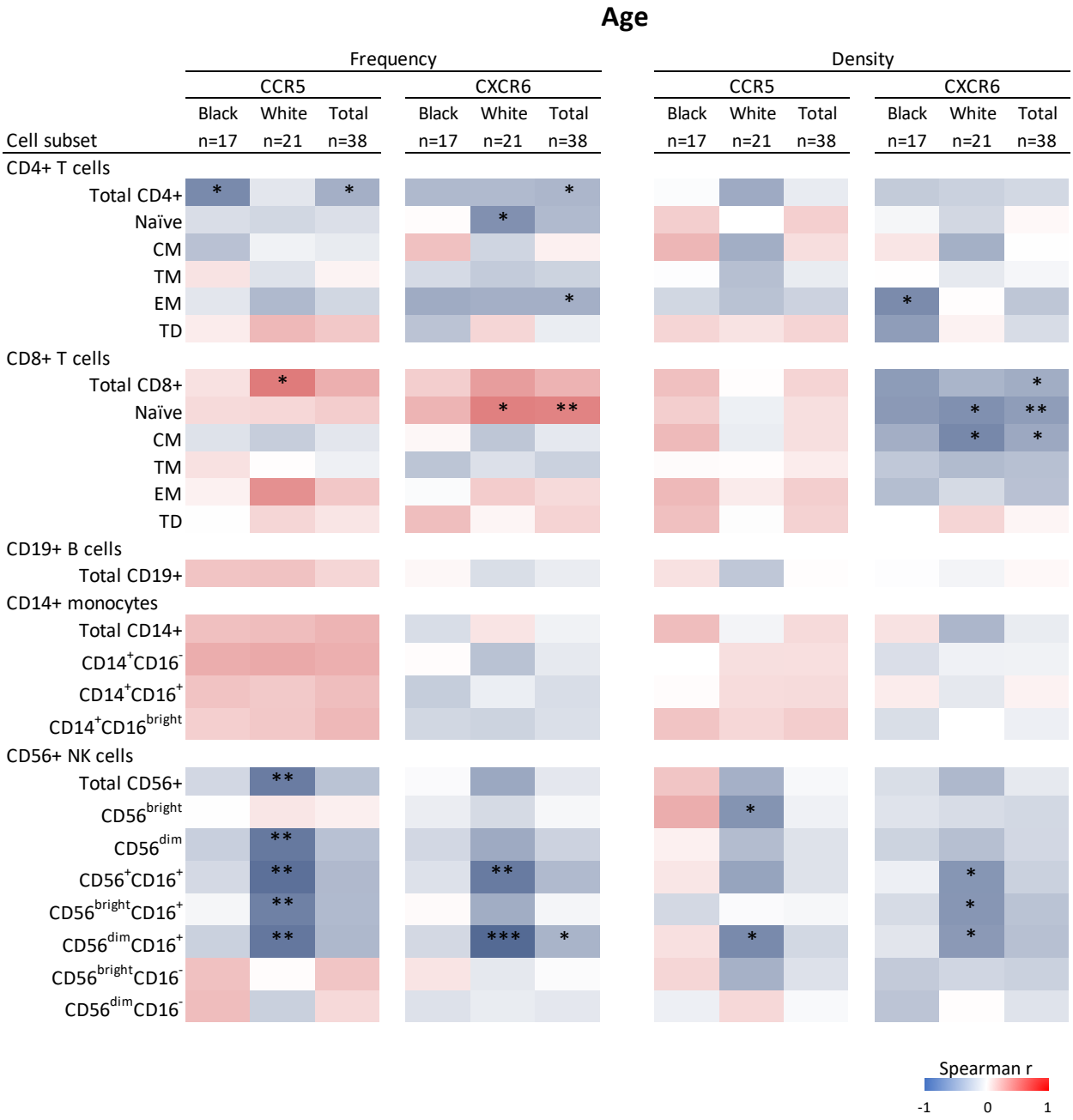


Figure 5.2 - Heatmap showing correlations between age and CCR5 and CXCR6 expression on (a) total CD4+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD4+ T cell subsets (b) total CD8+ T cells and naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets, (c) CD19+ B cells, (d) total CD14+ monocytes and monocyte subsets and (e) total CD56+ NK cell subsets. Correlations are shown for both frequency of expression (left 2 columns) and density (right 2 columns). The darker the colour, the stronger the correlation. * p<0.05; ** p<0.01; *** p<0.001. darker the colour, the stronger the correlation. * p<0.05; ** p<0.01; *** p<0.001.

5.4 Discussion

Females tend to mount stronger innate and adaptive immune responses than males, which results in a faster clearance of pathogens, but also contributes to an increased susceptibility to inflammatory and autoimmune diseases (269). Following stimulation of peripheral blood mononuclear cells (PBMCs) *in vitro*, women were found to have a higher number of activated CD4+ and CD8+ T cells compared to men (270,271). In women, CD4+ and CD8+ T cells had a significantly higher level of immune activation-associated phenotypes in peripheral blood and in gut associated lymphoid tissue (271). While HIV-1 loads in untreated women are up to 40% lower than those in males, the higher immune activation seen in women is linked to the subsequent faster disease progression and premature aging observed in chronically infected women, compared with men, for the same level of viral replication (251,272).

Sex differences had a minimal impact on CCR5 expression. Where there were differences, results were population-specific and primarily observed on the CD8+ T cell subsets. No differences were seen with regards to CCR5 density on any cell type. In the total group, females had a higher frequency of CCR5-expressing terminally differentiated CD8+ T cells compared to males. Since CCR5 is an indicator of activation on CD8+ T cells, this finding could be as a result of increased immune activation seen in women compared to men. However, in black individuals, females had a significantly lower frequency of CCR5-expressing central memory CD8+ T cells compared to males, which was also reflected in the total group due to the strength of the finding in the black population. White females had a significantly lower percentage of CCR5-expressing $CD56^{bright}CD16^{-}$ NK cells compared to white males.

Sex differences were observed predominantly in the frequency of CXCR6-expressing cells rather than CXCR6 cell density and only in the white population group. Males had a higher frequency of CXCR6+ CD4+ T cells (naïve, transitional memory and effector memory), postulated to be protective in HIV-2 and SIV pathogenesis (122,123), and had a lower frequency of CXCR6+ transitional memory CD8+ T cells compared to females. It has been shown in peripheral blood that 35–56% of CXCR6+ CD4+ T cells are type 1 helper (Th1) cells and 60–65% of CXCR6+ CD8+ T cells are type 1 cytotoxic (Tc1) cells, with very few CXCR6+ cells being type 2 cells (213). Th1 and Tc1 cells, as well as NK cells, secrete interferon-γ (IFN-γ) and Tc1 cells kill target cells by releasing cytotoxic molecules, such as granzymes and perforin, into the

immunological synapse (273–277). Almost all CXCR6+ Tc1 cells have the cytotoxic effector phenotype (213). CXCR6+ CD4+ T cells have been shown to be responsible for the production of the inflammatory Th1 and Th17 effector cytokines, mediating chronic inflammatory responses (278).

Many studies have failed to address sex differences in HIV-1 acquisition and progression. In this study, sex differences had an impact on CCR5 and CXCR6 expression, however this varied across the two population groups studied. It would be interesting to know whether these population-specific sex differences in CCR5 and CXCR6 expression translate into differential HIV-1 control between the two population groups.

Progression to AIDS tends to occur significantly faster in older infected individuals (279,280). Aging has previously been linked with an increased CCR5 expression on T cells in both mice and humans (264–266,281). In black individuals, aging was associated with a significant decrease in the frequency of CCR5-expressing CD4+ T cells, which was unexpected. However, due to our small sample size, it is hard to draw conclusions as to whether this is due to true ethnic differences without assessing this finding in bigger cohorts.

The percentage of CCR5 expression on CD8+ T cells has been shown to increase with age in the peripheral blood, lymph node, spleen, and mucosal gut-associated lymphoid tissues, likely in response to antigen exposure, inflammation, or maturation of immune responses(282). In white individuals, as well as in the total group, aging associated with an increase in the frequency of CCR5-expressing CD8+ T cells. In agreement, this was also shown previously in a study by our group in another cohort of healthy controls, however only in the combined black and white population group (195).

Aging has been found to increase the population of $CD56^{dim}CD16^{+}$ NK cells while decreasing functional capacity and further contributing to systemic inflammation (283) and a recent study found a disruption of natural killer cell homing in aging individuals living with or without HIV-1 (284). In our study, only in white individuals, aging strongly significantly associated with a lower frequency of CCR5-expressing NK cells ($CD56^{dim}$, $CD56^{+}CD16^{+}$, $CD56^{bright}CD16^{+}$ and $CD56^{dim}CD16^{+}$ NK cells), and with a lower CCR5 density on $CD56^{bright}$ and $CD56^{dim}CD16^{+}$ NK cells. Since CCR5 expression on NK cells has been shown to integrally impact chemokinesis of

NK cells in response to cytokines in addition to redistributing NK cells to areas of active viral replication (222), the lower CCR5 expression seen on NK cells of older individuals in our study could represent a mechanism of NK cell homing disruption in older individuals. Older individuals also exhibit decreased NK cell activity, which has been shown to be associated with an increased incidence and severity of multiple diseases (285,286). How these population-specific results translate to potential variable HIV-1 control across ethnicities requires further investigation.

In our study, aging affected CXCR6 expression on all cell types except for B cells and CD14+ monocytes. In both population groups and in the combined group, older age was inclined to correlate with lower CXCR6 expression except on CD8+ T cells, where older age correlated with a higher frequency of CXCR6-expressing naïve CD8+ T cells (the opposite was seen with regards to CXCR6 density on CD8+ T cells). This result only reached significance in the white population group and in the total group. In agreement with these results, circulating CXCR6+ CD8+ T cells have been shown to significantly increase with advancing age (287). An increased frequency of CXCR6+ CD8+ T cells in older individuals may be indicative of a pro-inflammatory phenotype, potentially rendering this group susceptible to hyper-inflammatory immune responses, harmful with regards to HIV-1 acquisition since lower levels of immune activation are strongly associated with resistance to HIV in people without HIV-1 who are exposed to HIV-1 (288,289).

Both CD4+ and the CD8+ T cells of older individuals have been shown to secrete a significantly larger amount of IFN-γ after activation (290). Since it has been shown that 80–90% of CXCR6+ CD8+ T cells were positive for intracellular granzyme A and are efficient in IFN-γ production and these cells are increased with age in our study, it might offer a mechanism for the increased IFN-γ production seen in older individuals (213). Older individuals who acquire HIV-1 have a quicker disease onset and worse clinical outcomes, which has been associated with increased CCR1, CCR2, CCR3, CCR4 and CCR5 expression in PBMCs and CD4+ T cells (264). Perhaps CXCR6 also plays a role in this phenomenon.

Exclusively in the white population group, there were strongly significant negative correlations with aging and both the frequency of CXCR6-expressing cells and CXCR6 density on only the CD16+ subsets of NK cells. Since CD16+ NK cells exhibit IFN-γ production and cytolytic activity

as an immediate innate immune response against pathogens (235), this may translate to a less effective innate immune response in older individuals, which has been described previously (291).

In humans, the majority of CXCR6-expressing NK cells are found in the liver, and to a lesser extent in the spleen, bone marrow, and lymph nodes (132,292). Liver disease is a frequent cause of death among people living with HIV-1 (293–295). It has been shown that the memory response of hepatic NK cells, specifically to haptens and viruses, depend on CXCR6, allowing hepatic NK cells to develop adaptive immunity to structurally diverse antigens (132). Evidence is suggestive that older individuals living with HIV-1 may be at a higher risk of liver disease (296). Although viral hepatitis is the main contributor to liver-related deaths among people living with HIV-1, the proportion of liver disease attributed to non-alcoholic fatty liver disease (NAFLD) is increasing (297). NAFLD seems to follow racial/ethnic disparities, with Africa having the lowest prevalence (298). It would be interesting to determine if increased liver disease outcomes in older people living with HIV-1 is directly associated with lower CXCR6 expression on NK cells and if CXCR6 expression could be contributing to the varying outcomes of liver disease seen with regards to ethnicity.

Age tended to correlate with CCR5 and CXCR6 expression in similar patterns across cell types, except on B cells and monocytes, where age tended to positively correlate with CCR5 expression and negatively correlate with CXCR6 expression. While age correlated similarly with percentage of CCR5 and CXCR6-expressing CD8+ T cells, age tended to positively correlate with CCR5 density and negatively correlate with CXCR6 density on CD8+ T cells.

Many studies have failed to address sex and age differences in HIV-1 acquisition and progression. In this study, in two ethnically distinct healthy HIV-1 uninfected populations, both sex and age had an impact on CCR5 and CXCR6 expression, and this varied widely across the two population groups studied. Whether these population-specific sex and age differences in CCR5 and CXCR6 expression translates into differential HIV-1 control between the two population groups requires further investigation. This study emphasises the importance of taking sex and age into consideration in studies involving disease and disease outcomes.

CHAPTER SIX

6. Select *CCR5* SNPs have varying associations with CCR5 expression levels in two ethnically distinct South African population groups

6.1 Introduction

Expression levels of the chemokine receptor CCR5, which acts as a major HIV-1 coreceptor in addition to being an integral protein in immunological functioning, affect numerous aspects of HIV-1 pathogenesis and outcomes of disease. Individuals have highly variable expression levels of CCR5 (299), which may in part be explained by polymorphisms in the *cis*-regulatory regions of *CCR5.* The rs553615728 -4223 C>T single nucleotide polymorphism (SNP) disrupts a cytidine phosphate guanidine (CpG) dinucleotide in the *cis*-region of *CCR5,* CpG-41, a binding site where DNA methylation occurs, and has been uniquely found in individuals from southern Africa (115). The frequency of the -4223 C>T SNP was found to be greater in long-term non-progressors and HIV-1 controllers when compared to progressors and this SNP was overrepresented in female sex workers resisting HIV compared with those who subsequently acquired HIV (115).

The *CCR5* −2459 G>A SNP (rs1799987) has been linked to differences in CCR5 expression levels on CD14+ monocytes, with the density of CCR5 being lower on unstimulated CD14+ monocytes from healthy Caucasian individuals with the –2459 GG and AG genotypes compared to the AA genotype (178), and the AA genotype has been associated with a faster rate of progression to AIDS (117–119). In our prior work (Chapter 3), the -2459G>A SNP was significantly underrepresented in HIV-1 controllers compared to progressors. The -2459 SNP is common to the *CCR5* HHE, HHF and HHG haplotypes (which have been reported to be high promoter activity haplotypes (104,106); deleterious in the context of HIV-1). In our previous work (Chapter 3), individuals heterozygous for the -2459 SNP were significantly less likely to control HIV-1 in total group comparisons as well as in elite controllers and viraemic controllers compared to progressors and possession of the minor allele was significantly underrepresented in controllers when compared to progressors. In a cohort of self-identified white and black patients, a study observed that the -2459 G>A SNP had a strong association with the time taken to achieve virologic success of highly active anti-retroviral therapy (HAART) in black but not in white patients ($p=0.04$), and that this association increased with stronger African ancestry (179).

The 3'UTR plays a major role in gene expression and regulation by influencing the localization, stability, export, and translation efficiency of an mRNA (180). In prior work (Chapter 3), we showed that the 3'UTR rs746492 (+2919) T>G SNP significantly associated with HIV-1 disease progression (300). The +2919T>G SNP was in strong LD with the CCR5 promoter HHE, HHF*1, HHF*2 and HHG*1 haplotypes (deleterious with regards to HIV-1) and the -2459 SNP (common to these haplotypes), thus begging the question as to which polymorphism is functionally driving the deleterious effect on HIV-1 control in black South Africans. We found that the strongest association with progression was when looking at possession of the +2919T>G SNP in the dominant mode ($p=0.001$; $p_{bonferroni}=0.016$). To our knowledge, no other studies have associated this SNP with increased risk of HIV-1 disease acquisition or more rapid progression of HIV-1.

Genome wide association studies (GWAS) have shown strong associations with HIV-1 outcomes for the rs1015164 G>A polymorphism. This SNP marks expression of the antisense long non-coding RNA (lncNRA) CCR5AS (121). CCR5AS expression levels in CD4+ T cells significantly positively correlated with levels of CCR5 mRNA expression (117). The rs1015164 SNP had a genome-wide effect independent of other SNPs in the region, including *CCR5Δ32* (121).

This study was thus undertaken to investigate the direct association between *CCR5* -4223 C>T (rs553615728), -2459 G>A (rs1799987), +2919 T>G (rs746492) and rs1015164 G>A SNPs and expression levels of CCR5 on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells in two ethnically distinct South African population groups. It is important to evaluate polymorphisms in chemokine ligands and receptors as they may have an impact on HIV-1 disease transmission and progression through mechanisms beyond viral entry. Given that different ethnicities exhibit remarkable differences in host genetic backgrounds, this will provide information that may assist with understanding the difference in HIV-1 control mechanisms and the varying expression levels of CCR5 in different population groups.

6.2 Materials and Methods

6.2.1 Sample population

Refer to Chapter 2, Materials and Methods section 2.1. Briefly, black (n=17) and white (n=21) South Africans without HIV-1 were recruited and were age- and sex-matched.

6.2.2 Single nucleotide polymorphism (SNP) genotyping

Individuals were genotyped for *CCR5* -4223 C>T (rs553615728), -2459 G>A (rs1799987), +2919 T>G (rs746492) and rs1015164 G>A SNPs as described in Chapter 2, Materials and Methods section 2.6.

6.2.3 Flow cytometry

CCR5 expression on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells for each individual was determined as described in Chapter 2, Materials and Methods section 2.7 and assessed as outlined in Materials and Methods section 2.9.

6.2.4 Statistical analyses

Statistical analyses were performed as described in Chapter 2, Materials and Methods section 2.10.

6.3 Results

6.3.1 Allelic and genotypic representation of *CCR5* SNPs

The frequency of the CCR5 SNPs assessed in this study were represented differently depending on the population group studied. Genotypic and allelic frequencies for each SNP in black and white South Africans are shown in Table 6.1.

Table 6.1 - Genotypic and allelic frequencies of the -4223 C>T, -2459 G>A, +2919 T>G and rs1015164 G>A polymorphisms in black and white South Africans.

		CCR5 polymorphism			
		CCR5 -4223 C>T	**CCR5 -2459 G>A**	**CCR5 +2919 T>G**	**rs1015164 G>A**
Black South Africans		(n=34)	(n=28)	(n=34)	(n=34)
Allelic	WT	33	17	20	34
	Mt	1	11	14	0
		(n=17)	(n=14)	(n=17)	(n=17)
Genotypic	WT/WT	16	7	7	17
	WT/Mt	1	3	6	0
	Mt/Mt	0	4	4	0
White South Africans		(n=42)	(n=36)	(n=42)	(n=42)
Allelic	WT	42	21	24	32
	Mt	0	15	18	10
		(n=21)	(n=18)	(n=21)	(n=21)
Genotypic	WT/WT	21	8	8	12
	WT/Mt	0	5	8	8
	Mt/Mt	0	5	5	1

6.3.2 *CCR5* -4223 C>T SNP and CCR5 expression

As expected, due to the extremely low frequency of this SNP and its exclusivity to the southern African black population, only 1 black individual in our study was heterozygous for the -4223 SNP and thus association to CCR5 expression could not be assessed. It is interesting with how rare this SNP is that it was still found at a frequency of 0.06% in our small sample of black South African individuals (n=17).

6.3.3 *CCR5* -2459 G>A SNP and CCR5 expression

As reported on Ensembl's database (301), the ancestral allele of the *CCR5* -2459 SNP is G. It is a commonly reported SNP in both European and African population groups. Six individuals (black n=3; white n=3) were unable to be genotyped with our assay due to indels in the region and so were excluded from analyses.

A summary of significant associations between the -2459 SNP and CCR5 expression can be seen in Figure 6.1. Results were population-specific. Of note, no results remained significant after correction for multiple comparisons.

In black individuals, the GG genotype tended to associate with a lower CCR5 expression compared to the AA genotype (naïve CD8+ T cells, $CD14^{+}CD16^{bright}$ monocytes and $CD56^{dim}CD16^{-}$ NK cells) except for total CD4+ T cells and central memory CD8+ T cells where the GG genotype associated with a higher CCR5 expression compared to the GA or AA genotype. In white individuals, the -2459 GG genotype associated with higher CCR5 expression on total CD8+ T cells, transitional memory CD8+ T cells, B cells, and $CD56^{bright}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells. In the total group, the -2459 GG genotype associated with higher CCR5 expression on total CD4+ T cells, effector memory CD4+ T cells and central memory CD8+ T cells.

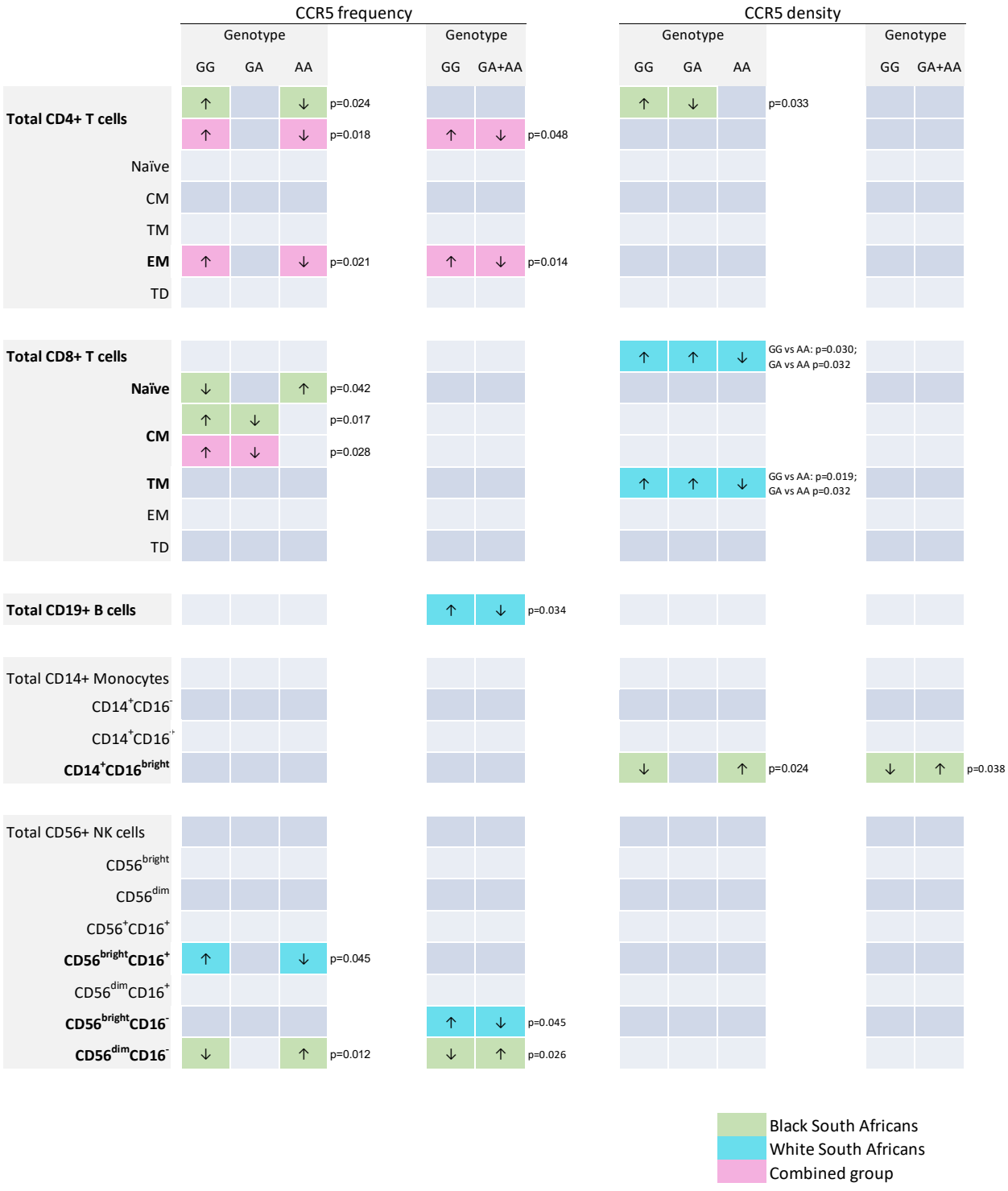


Figure 6.1 - Summary of significant changes to percentage of CCR5-expressing CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells associated with -2459 SNP genotype. ↑ indicates an increased expression and ↓ indicates a decreased expression associated with the respective genotype. Cell types with significant expression changes are bolded. p values for significant comparisons are shown. CM: central memory; TM: transitional memory; EM: effector memory; TD: terminally differentiated.

6.3.4 *CCR5* +2919 T>G SNP and CCR5 expression

As reported on Ensembl's database (301), the ancestral allele of the *CCR5* +2919 SNP is T. However, while we used the T allele as our wild-type reference allele and TT as our wild-type reference genotype, because the G allele is found in a high frequency in both African and European population groups (301), we found it informative to carry out analyses by evaluating the effect of the allele in two modes, i.e., TT vs TG+GG and GG vs TG+TT.

6.3.4.1 Frequency of CCR5 expression and the +2919 SNP

The *CCR5* +2919 SNP associated with frequency of CCR5 expression across both population groups and across all cell types. A summary of significant associations with the +2919 SNP and CCR5 expression (frequency) can be seen in Figure 6.2.

6.3.4.1.1 CD4+ T cells

In black individuals, the +2919 TT genotype associated with a higher percentage of CCR5-expressing total CD4+ T cells compared to the GG genotype (p=0.042) and a higher percentage of CCR5-expressing effector memory CD4+ T cells compared to the TG genotype and the TG+GG genotype (p=0.035 and p=0.019, respectively). In the total group, the GG genotype associated with a significantly lower percentage of CCR5-expressing effector memory and terminally differentiated CD4+ T cells compared to the TT and TG genotype respectively (p=0.041 and p=0.033, respectively) and compared to the TG+TT genotype (p=0.049 and p=0.049, respectively). The strongest significant finding in the total group was the TT genotype associating with a higher percentage of effector memory CD4+ T cells compared to the TG+GG genotype (p=0.028). Consistently, the G allele associated with a lower percentage of CCR5 expression on CD4+ T cells, a known protective phenotype.

6.3.4.1.2 CD8+ T cells

With regards to percentage of CCR5-expressing CD8+ T cells, the +2919 GG genotype significantly associated with lower CCR5 expression across all cell subsets in a population-specific manner, except for central memory CD8+ T cells, where in black individuals only, the TG genotype associated with lower expression compared to the TT genotype (p=0.005). Also in black

individuals, the GG genotype associated with a significantly lower percentage of CCR5-expressing total CD8+ T cells compared to the TG genotype (p=0.010) and this was replicated more strongly in the total group, both compared to the TG genotype and the TG+TT genotype (p=0.002 and p=0.006, respectively).

In white individuals, the GG genotype associated with a lower percentage of CCR5-expressing naïve CD8+ T cells compared to the TT genotype and the TG+TT genotype (p=0.006 and p=0.011, respectively). The TG+GG genotype also associated with a lower percentage of CCR5-expressing naïve CD8+ T cells compared to the TT genotype (p=0.030). The GG genotype again associated with a lower percentage of CCR5-expressing transitional memory CD8+ T cells compared to both the TG genotype and the TG+TT genotype (p=0.045 and p=0.040, respectively). This finding was replicated and strengthened in the total group (p=0.007 and p=0.009, respectively). In the total group, the GG genotype associated with a significantly lower percentage of CCR5-expressing effector memory CD8+ T cells (p=0.028). This same pattern was seen in black individuals on terminally differentiated CD8+ T cells (p=0.010) and in the total group, TG associated with a significantly higher percentage of CCR5-expressing terminally differentiated CD8+ T cells compared to both the TT and GG genotype (p=0.010 and p=0.003, respectively), with a less significant higher percentage of CCR5-expressing terminally differentiated CD8+ T cells associating with the TG+TT genotype compared to the GG genotype (p=0.041).

6.3.4.1.3 B cells

In white individuals only, the GG genotype significantly associated with a lower percentage of CCR5-expressing B cells compared to the TT genotype and the TG+TT genotype (p=0.002 and p=0.006, respectively). Additionally, the TT genotype associated with a significantly higher percentage of CCR5-expressing B cells compared to the TG+GG genotype (p=0.030).

6.3.4.1.4 Monocytes

Only in the total group analyses, the +2919 TG genotype associated with a significantly higher percentage of CCR5-expressing $CD14^{+}CD16^{-}$ monocytes (vs GG; p=0.016), $CD14^{+}CD16^{+}$ monocytes and $CD14^{+}CD16^{bright}$ monocytes (vs TT; p=0.046 and p=0.023, respectively).

6.3.4.1.5 NK cells

In white South Africans only, the GG genotype associated with a significantly lower percentage of CCR5-expressing $CD56^{bright}$ NK cells compared to the TG and TG+TT genotype ($p=0.030$ and $p=0.025$, respectively.) The GG genotype also associated with a significantly lower percentage of CCR5-expressing $CD56^{bright}CD16^{+}$ NK cells compared to the TT genotype ($p=0.030$), the TG genotype ($p=0.045$) and the TG+TT genotype ($p=0.015$).

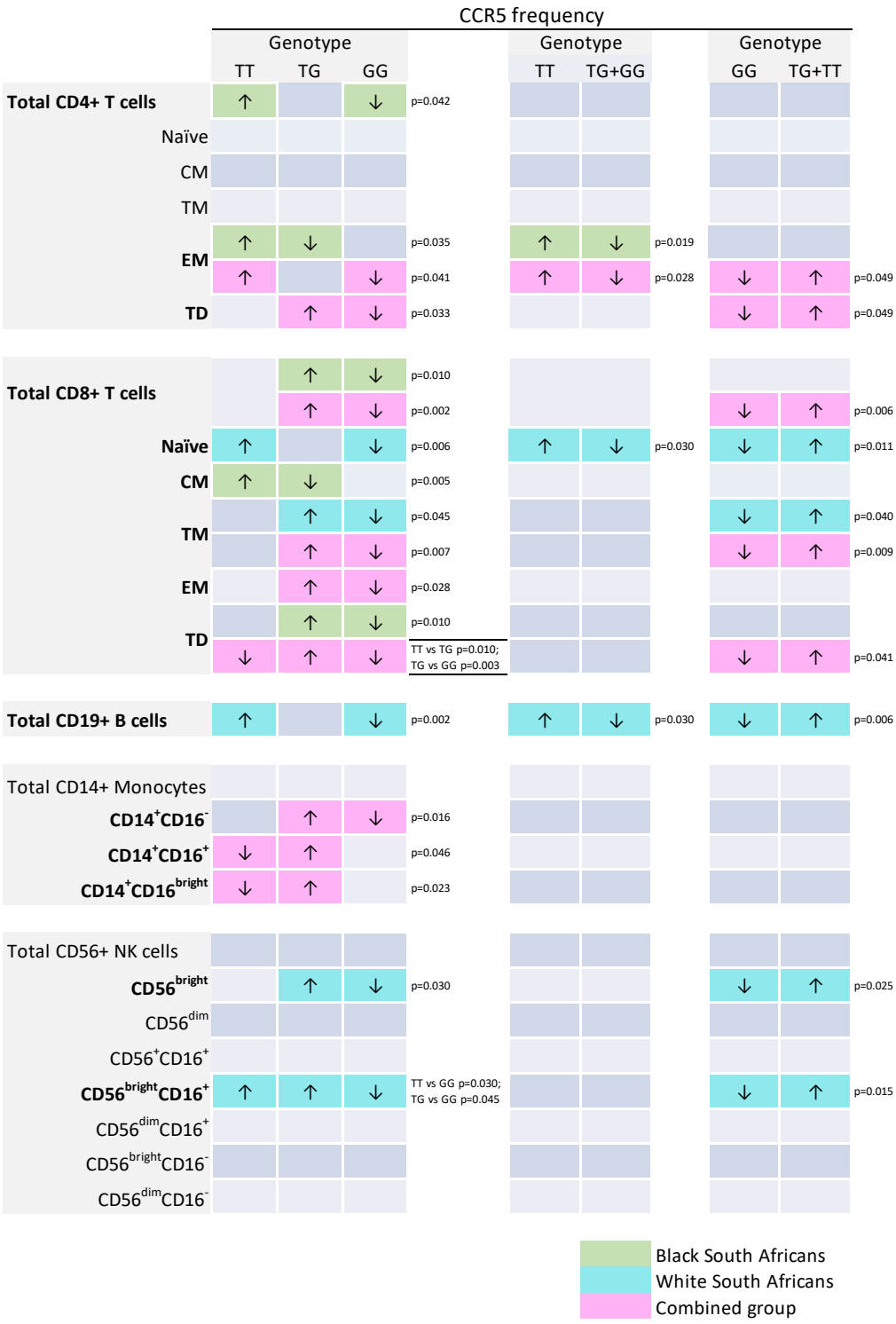


Figure 6.2 - Summary of significant changes to percentage of CCR5-expressing CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells associated with +2919 SNP genotype. ↑ indicates an increased expression and ↓ indicates a decreased expression associated with the respective genotype. Cell types with significant expression changes are bolded. p values for significant comparisons are shown. CM: central memory; TM: transitional memory; EM: effector memory; TD: terminally differentiated.

6.3.4.1.6 Correction for multiple comparisons

To reduce the rate of type I errors when conducting multiple comparisons, we used the false discovery rate (FDR) correction to see which comparisons remained significant. For the results that remained significant after FDR corrections, we also applied the more conservative Bonferroni-Dunn correction. These results and the q values (FDR-adjusted p values) and adjusted $p_{Bonferroni}$ values, where applicable, are shown in Figure 6.3.

All significant associations between the +2919 SNP and CCR5 expression in the black population group, on CD4+ T cell subsets, monocyte subsets and NK cell subsets were lost after multiple comparison testing.

Remaining significant, in the total group, the TG genotype associated with a significantly higher percentage of CCR5-expressing total CD8+ T cells and transitional memory CD8+ T cells compared to the GG genotype ($q=0.025/p_{Bonferroni}=0.037$ and $q=0.034$, respectively) and compared to both the TT and GG genotype for terminally differentiated CD8+ T cells ($q=0.039$ and $q=0.025$, respectively). In white individuals only, the GG genotype significantly associated with a lower percentage of CCR5-expressing B cells compared to the TT and TG+TT genotypes ($q=0.003/p_{Bonferroni}=0.005$ and $q=0.012/p_{Bonferroni}=0.012$, respectively) and the TG+GG genotype associated with a lower percentage of CCR5-expressing B cells compared to the TT genotype ($q=0.032$).

Overall, the +2919 TG genotype associated with a significantly higher percentage of CCR5-expressing total CD8+ T cells, transitional memory and terminally differentiated CD8+ T cells compared to the GG genotype and the +2919 GG genotype associated with a significantly lower percentage of CCR5-expressing B cells compared to the TT and TG+TT genotype, only in white South Africans.

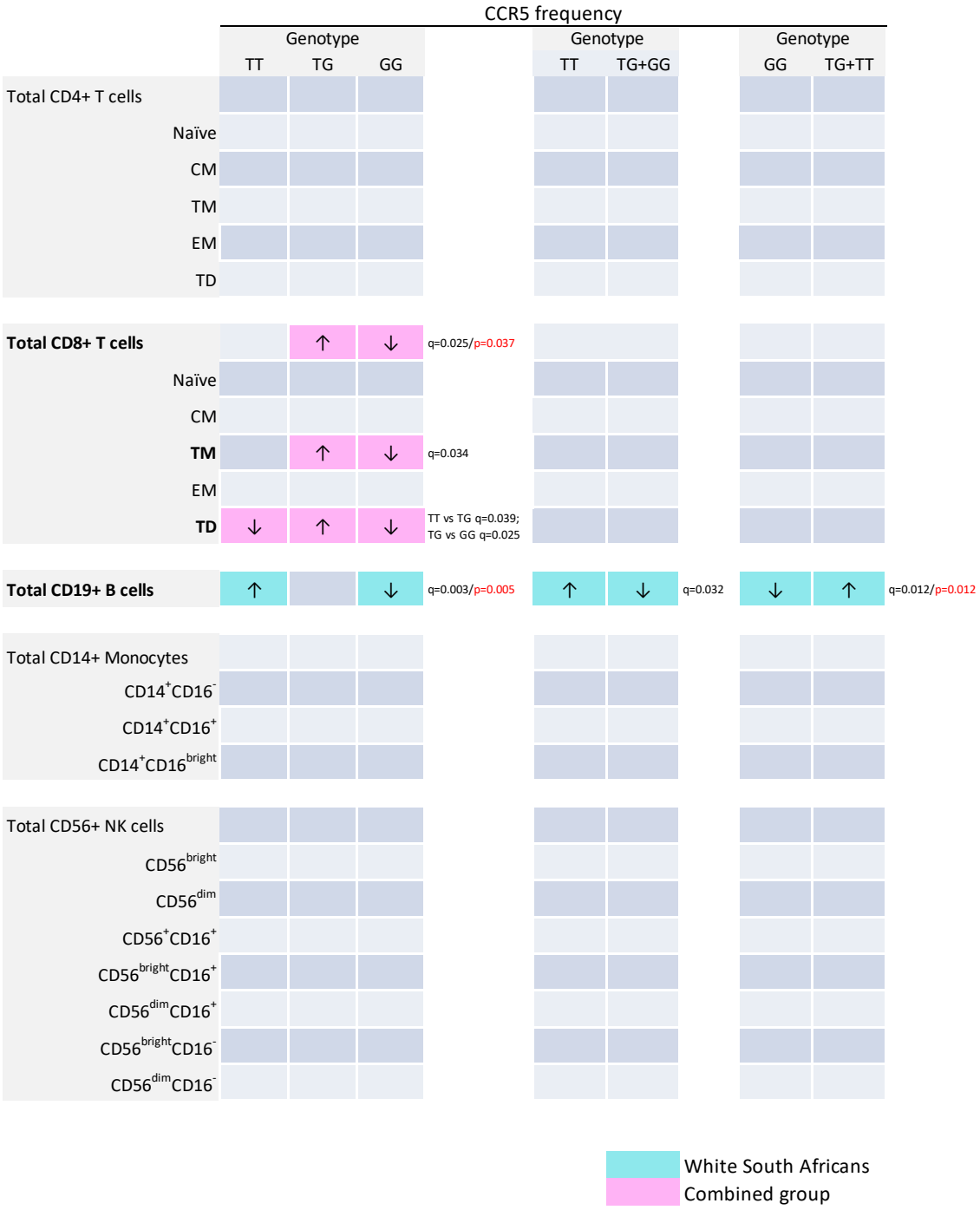


Figure 6.3 - Summary of significant changes to percentage of CCR5-expressing cells associated with +2919 SNP genotype surviving false discovery rate (FDR) and Bonferroni multiple comparison corrections. ↑ indicates an increased expression and ↓ indicates a decreased expression associated with the respective genotype. Cell types with significant expression changes are bolded. q values for post-FDR significant comparisons are shown. p values in red survived the more conservative Bonferroni correction testing. CM: central memory; TM: transitional memory; EM: effector memory; TD: terminally differentiated.

6.3.4.2 CCR5 density and the +2919 SNP

The *CCR5* +2919 SNP associated with CCR5 density on CD4+ and CD8+ T cell subsets in white individuals and in the total group and on NK cell subsets in black individuals. A summary of significant associations with the +2919 SNP and CCR5 expression (density) can be seen in Figure 6.4.

6.3.4.2.1 CD4+ T cells

The +2919 GG genotype significantly associated with lower CCR5 density on total CD4+ T cells compared to the TT genotype in the total group (p=0.041) and on transitional memory CD4+ T cells compared to the TT and TG+TT genotype in white individuals (p=0.045 and p=0.025, respectively).

6.3.4.2.2 CD8+ T cells

In white individuals and in the total group, the +2919 GG genotype associated with significantly lower density on every CD8+ T cell subset except naïve CD8+ T cells compared to either only the TG genotype (total group: terminally differentiated), the TG and TG+TT genotype (white: central memory, effector memory, terminally differentiated) or the TG, TT and TG+TT genotype (white: total CD8+ T cells, transitional memory; total group: total CD8+ T cells, central memory, transitional memory, effector memory).

6.3.4.2.3 NK cells

Only in black individuals, the TT genotype associated with significantly higher CCR5 density on $CD56^{bright}CD16^{+}$ NK cells compared to the TG genotype and TG+GG genotype (p=0.008 and p=0.033, respectively).

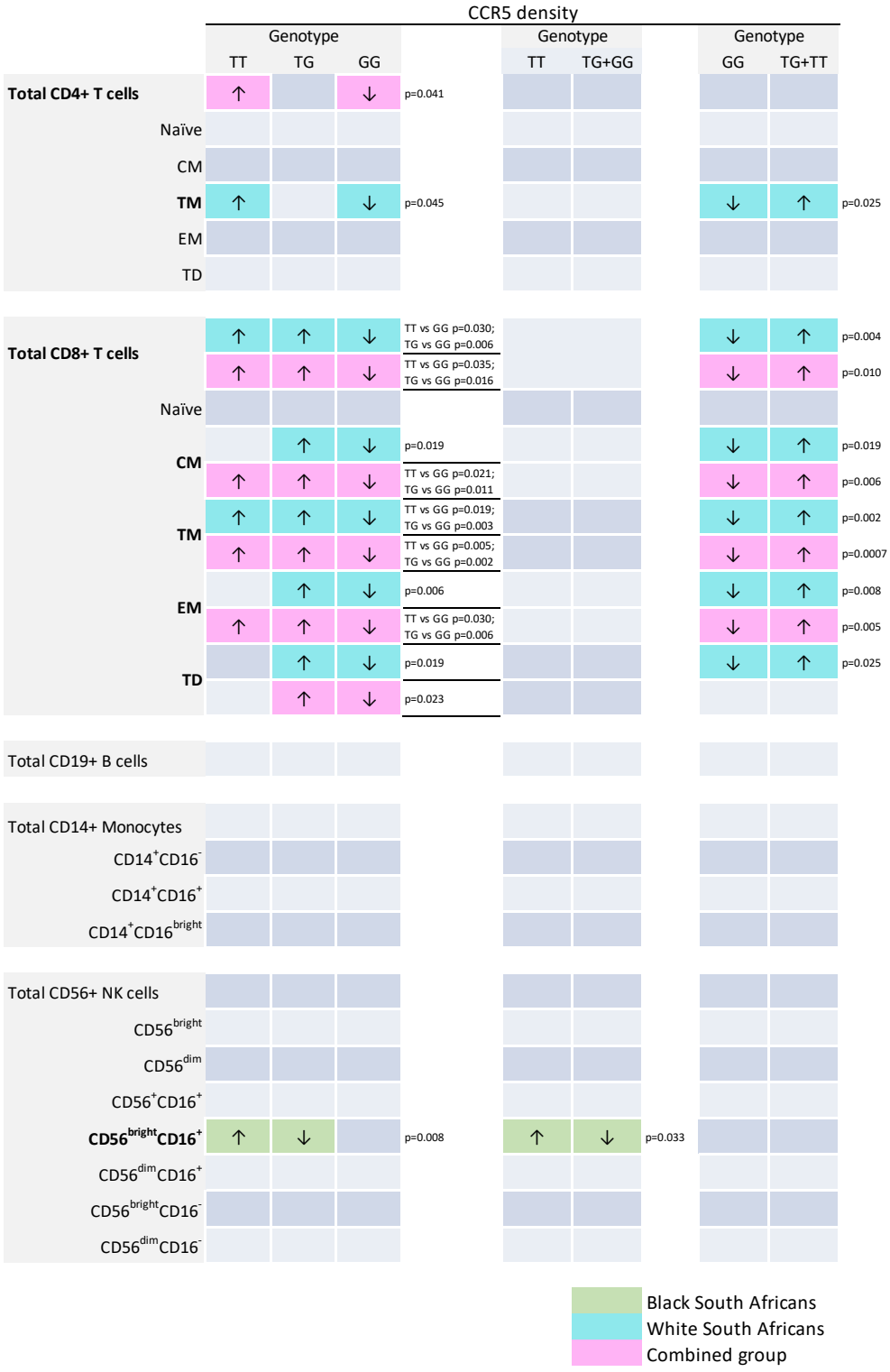


Figure 6.4 - Summary of significant changes to CCR5 density on CD4+ T cells, CD8+ T cells, B cells, monocytes and NK cells associated with +2919 SNP genotype. ↑ indicates an increased expression and ↓ indicates a decreased expression associated with the respective genotype. Cell types with significant expression changes are bolded. p values for significant comparisons are shown. CM: central memory; TM: transitional memory; EM: effector memory; TD: terminally differentiated.

6.3.4.2.4 Correction for multiple comparisons

We once again corrected for multiple comparisons using FDR correction to see which comparisons remained significant. For the results that remained significant after FDR corrections, we also applied the more conservative Bonferroni-Dunn correction. These results and the q values (FDR-adjusted p values) and adjusted $p_{Bonferroni}$ values, where applicable, are shown in Figure 6.5.

All significant associations between the +2919 SNP and CCR5 density in the black population group, on CD4+ T cell subsets and NK cell subsets were lost after multiple comparison testing.

Remaining significant were all of the CD8+ T cell findings where the GG genotype associated with lower CCR5 density compared to the TG and TG+TT genotype (all subsets except naïve CD8+ T cells) and compared to the TT genotype on central memory (white only) and transitional memory CD8+ T cells (white and total group). There were four comparisons that remained significant after Bonferroni-Dunn correction: total group TT vs GG on transitional memory CD8+ T cells ($p_{Bonferroni}$=0.028), white individuals GG vs TG+TT on total CD8+ T cells ($p_{Bonferroni}$=0.050) and total group and white individuals GG vs TG+TT on transitional memory CD8+ T cells ($p_{Bonferroni}$=0.022 and $p_{Bonferroni}$=0.009, respectively).

Overall, the +2919 TG and TG+TT genotype associated with significantly higher CCR5 density on all CD8+ T cell subsets except naïve CD8+ T cells compared to the GG genotype.

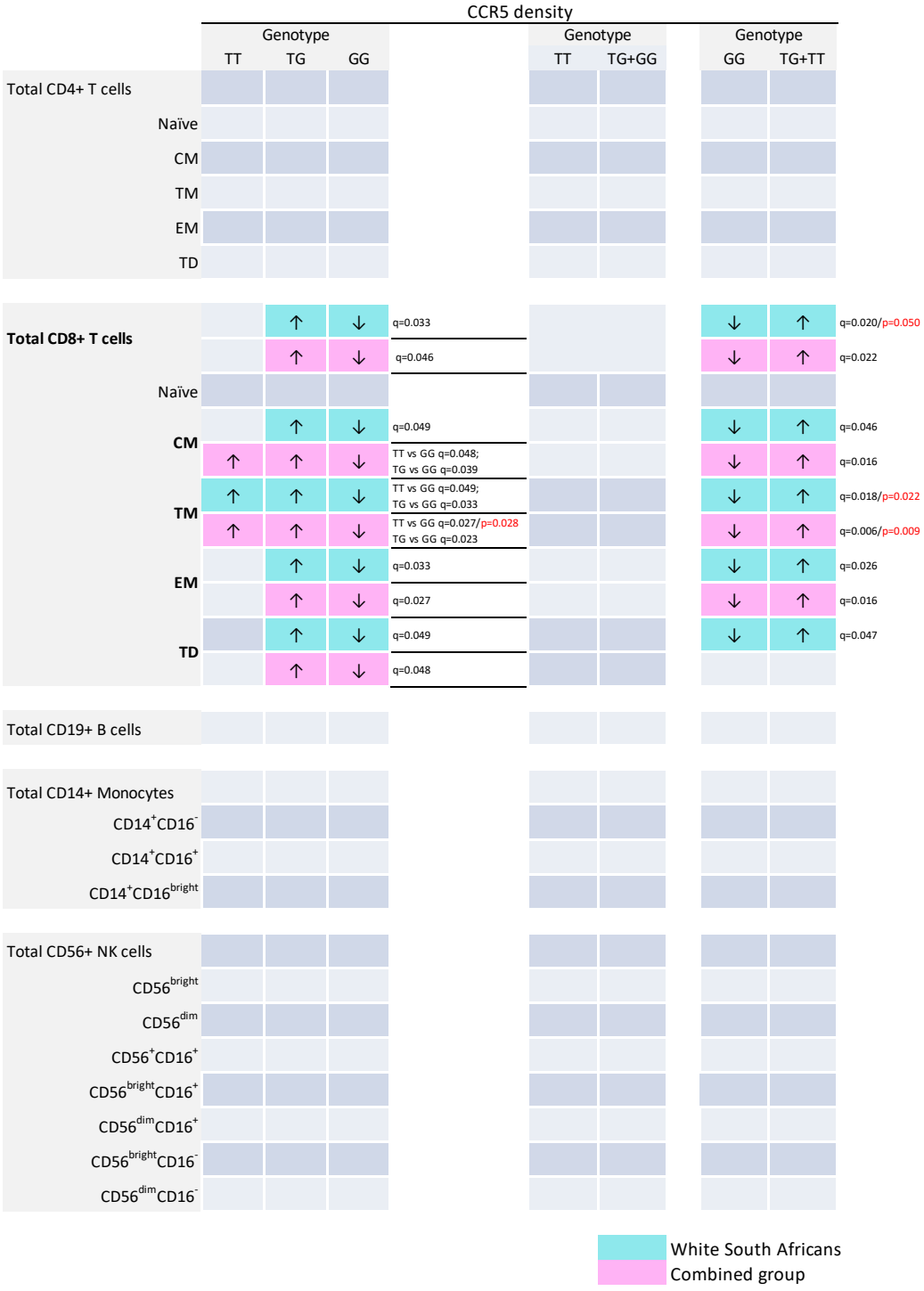


Figure 6.5 - Summary of significant changes to CCR5 density associated with +2919 SNP genotype surviving false discovery rate (FDR) and Bonferroni multiple comparison corrections. ↑ indicates an increased expression and ↓ indicates a decreased expression associated with the respective genotype. Cell types with significant expression changes are bolded. q values for post-FDR significant comparisons are shown. p values in red survived the more conservative Bonferroni correction testing. CM: central memory; TM: transitional memory; EM: effector memory; TD: terminally differentiated.

A visual representation of the associations between +2919 SNP genotypes and CCR5 expression on CD8+ T cells post FDR-correction testing can be seen in Figure 6.6 and Figure 6.7.

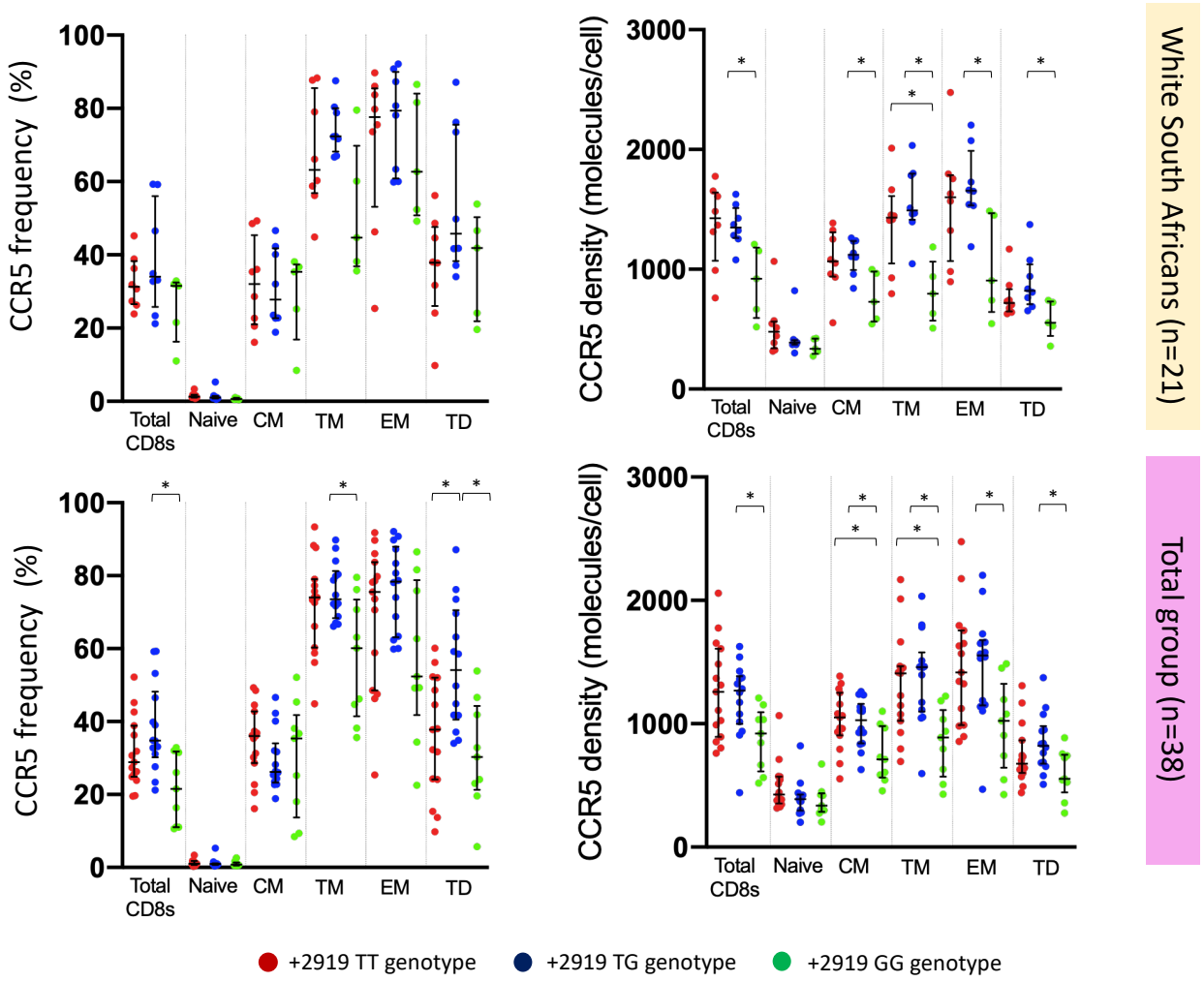


Figure 6.6 - Frequency (left) and density (right) of CCR5 expression according to the +2919 SNP genotypes for total, naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets in white South Africans and in the total group. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles. Asterisks represent a significant difference between groups, according to FDR testing.

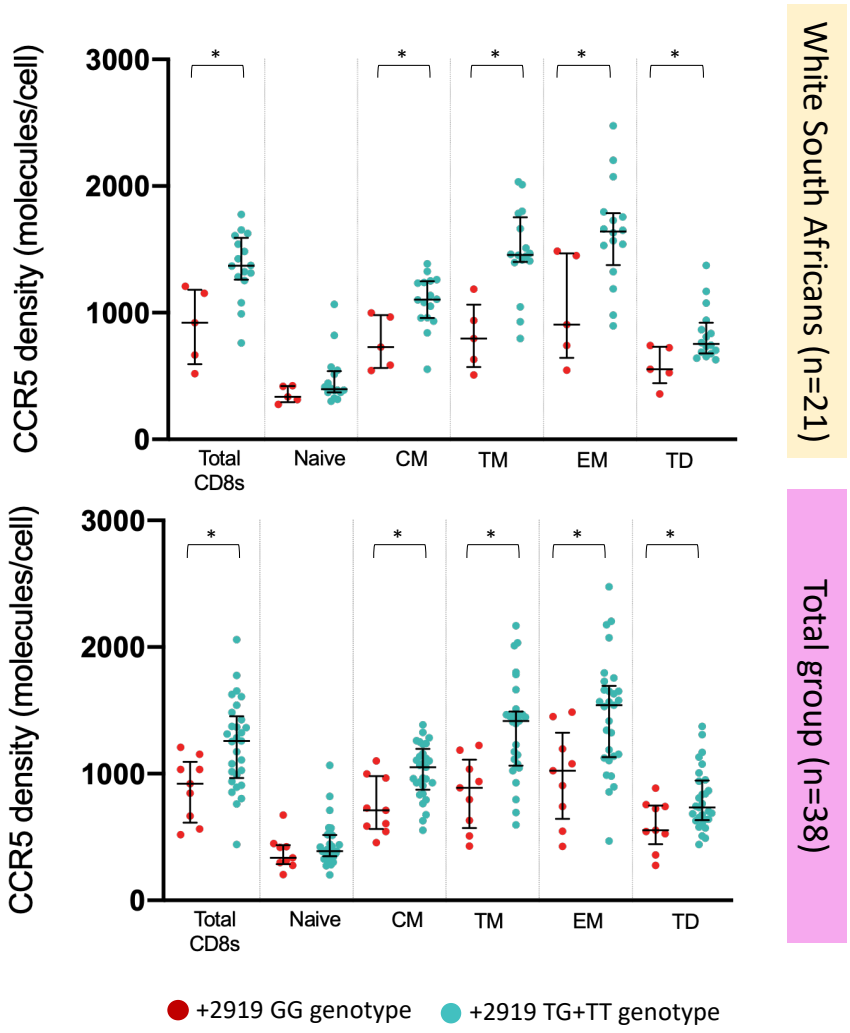


Figure 6.7 - +2919 SNP genotype (GG vs TG+TT) and CCR5 density on total, naïve, central memory (CM), transitional memory (TM), effector memory (EM), and terminally differentiated (TD) CD8+ T cell subsets in white South Africans and in the total group. Each symbol represents an individual. Horizontal lines and error bars represent the median, 25th and 75th percentiles. Asterisks represent a significant difference between groups, according to FDR testing.

6.3.5 *CCR5* rs1015164 G>A SNP and CCR5 expression

As reported on Ensembl's database (301), the ancestral allele of the rs1015164 SNP is G. Since the rs1015164 SNP minor allele was only detected at a frequency of 5% in Africans (n=661) (301), it is unsurprising that it was not detected in any of the 17 black South Africans in our study.

Because only one individual had the AA genotype, we analysed according to dominant mode i.e., GG vs GA+AA. In our study, the rs1015164 SNP did not associate with CCR5 expression (frequency or density) on any cell type either in white South Africans or in the total group.

6.4 Discussion

In this study, we investigated the association between four individual *CCR5* SNPs (-4223 C>T [rs553615728], -2459 G>A [rs1799987], +2919 T>G [rs746492] and rs1015164 G>A SNPs) and expression levels of CCR5 on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells in two ethnically distinct South African population groups. Of the four SNPs, only the +2919 T>G SNP resulted in significant associations after correction for multiple comparisons testing, with the -4223 SNP not being able to be assessed and the rs1015164 SNP not associating with CCR5 expression at all in our study. The -2459 SNP had population-specific associations with CCR5 expression. The AA genotype has been linked to a higher CCR5 density on CD14+ monocytes of healthy individuals (178) and this finding was replicated in our study on $CD14^{+}CD16^{bright}$ monocytes. The implication of these findings would need to be assessed further with a bigger sample size.

In previous work (Chapter 3), we identified that the +2919 T>G SNP in the dominant mode and +2919 T>G SNP heterozygosity were significantly underrepresented in total controllers and viraemic controllers compared to progressors. Whereas we found that the +2919 SNP in dominant mode (TT vs TG+GG) was a strong predictor of HIV-1 progression (Chapter 3), we did not see any associations with CCR5 expression in this mode, except for an increased percentage of CCR5-expressing B cells in white individuals only.

The majority of significant associations were found when we analysed CCR5 expression comparing the GG genotype to the TG+TT genotype (with the GG genotype associating with a lower CCR5 expression). Interestingly, in this study, even with a small sample size, we found that the +2919

TG genotype significantly associated with higher CCR5 expression (both with regards to frequency and density) on subsets of CD8+ T cells, however only in white individuals and in the total group.

Ethnic differences seen may be due to the varying linkage disequilibrium (LD) patterns present in different populations, since different ethnicities exhibit diverse haplotype heterogeneity at multiple loci (302–305). Whether the sample size of black individuals in this study is too small to result in significant associations or if this is a population-specific finding needs to be further assessed with bigger cohorts.

CCR5 also acts a marker of cellular activation (95,195,205). HIV-1 controllers are more likely to sustain long-term memory and effector potential CD8+ T cell responses compared to progressors, allowing for an increased ability to kill infected cells prior to progeny virion production (306,307). Effector memory CD8+ T cells are characterised by a high cytotoxic capacity (308). As HIV/SIV disease progresses, HIV-specific CD8+ T cell cytotoxicity decreases considerably until they no longer exert a suitable antiviral response (309,310). Chronic immune activation as a result of HIV-1 disease is linked to a faster disease progression to AIDS (311,312). Indeed, one of the greatest predictors of HIV-1 progression is hyperactivation of T cells (191,192,312). Higher activation levels of CD8+ T cells are associated with an acceleration of clinical HIV progression and a decline in CD4+ T cells in ART-naïve patients (58,191,313,314). In addition, T cell activation can be used to differentiate pathogenic and nonpathogenic SIV (155). There is a wide range of immune activation levels reported in individuals (227).

The majority of our significant findings were with regards to CCR5 density on CD8+ T cells. There are a vast number of studies demonstrating the importance of CCR5 receptor density in the context of HIV-1 acquisition and exhibiting that the amount of CCR5 expressed on the cell surface directly influences an individual's susceptibility to HIV-1 (189,315,316). An increased CCR5 density in people living with HIV-1 is associated with higher viral loads, faster disease progression and poorer response to antiretroviral treatment (98,102,317).

This study reveals a potential mechanism for our findings in Chapter 3 where we found that *CCR5* 3'UTR +2919 SNP heterozygosity was overrepresented in progressors compared to HIV-1 controllers, even more strongly than the known deleterious *CCR5* HHE haplotype. Further

assessment of this SNP in bigger cohorts, as well as functional assays to measure expression variation with the various CCR5 SNP genotypes, would be beneficial to determine the true significance of these findings and to determine whether the +2919 SNP is merely a marker of high CCR5 expression or a polymorphism driving higher CCR5 expression.

CHAPTER SEVEN

7. The *CXCR6* rs2234355 and rs2234358 SNPs associate with altered CXCR6 expression levels in two ethnically distinct South African population groups in the absence of HIV-1

7.1 Introduction

Both the HIV-1 pathogen and the host have inherent variability that result in varying outcomes with regards to HIV-1 acquisition and disease progression (70). It is likely that host-mediated control is the main mechanism of viral suppression leading to HIV-1 control (77). There are multiple host genetic factors that have been implicated in HIV-1 control, including *CCR5Δ32, HLA-B*57* and *-B*27*, and *KIR3DL1* and *KIR3DS1* alleles (87,318–320). Differential gene expression has also been implicated in HIV-1 control (321). Expression quantitative trait loci (eQTL) studies have shown convincing relationships between genetic variants and gene expression (322).

While most HIV-1 strains use CCR5 and/or CXCR4, lower pathogenicity HIV-2 and SIV strains use CXCR6 as a major coreceptor (122–124,148). The preferential usage of CXCR6 instead of CCR5 has been hypothesized to be protective, resulting in the steering of virus to cells able to support viraemia without causing immunodeficiency (123). CXCR6 is thought to specifically be protective in the presence of viraemia, with a study showing that in long-term non-progressors without control of viral load, the *CXCR6* gene was strongly and significantly associated with control (134). The rs2234358 G>T polymorphism gave the strongest signal and this finding was replicated in three additional independent European studies (323–325). This SNP significantly associated with HIV-1 control in viraemic controllers (VCs), independent of the CCR2-CCR5 locus (134), and the rs2234358 TT genotype was found to be significantly underrepresented in VCs compared to both healthy controls and progressors (135). The rs2234358 SNP lies within the *CXCR6* gene in a region of chromosome 3 that is rich in genes encoding chemokine receptors, located 42 base pairs (bp) downstream from the CXCR6 termination codon, and is positioned 422 kb from the *CCR5* gene (326).

Another SNP implicated in HIV-1 control, the *CXCR6* rs2234355 SNP (*CXCR6-E3K*), located in the N-terminus of *CXCR6*, has been associated with increased survival with regards to

Pneumocystis carinii pneumonia (PCP) progression to death among African Americans living with HIV-1 (136) and has been shown to associate with faster virological control failure on ART (137). Interestingly, this SNP is highly prevalent in African Americans (44%) while being extremely rare in European populations (<1%), emphasizing the importance of population specific studies. In previous work from our research group, *CXCR6-E3K* SNP heterozygosity was significantly overrepresented in black South African VCs when compared to healthy controls and progressors (135). The *CXCR6 E3K* mutation has been predicted to result in a reduced surface expression of CXCR6 (138).

Prior work from our research group revealed that viraemic controllers had a highly significant overrepresentation of the combination of the absence of the deleterious rs2234358 TT genotype and the presence of advantageous rs2234355 heterozygosity (i.e., -358TT/+355GA) compared to healthy controls and progressors, suggesting an additive effect in natural control of HIV-1, specifically in the presence of detectable viraemia (50>VL<2000 RNA copies/ml plasma) (135).

This study was undertaken to investigate the direct association between the *CXCR6* rs2234358 and rs2234355 SNPs and expression levels of CXCR6 on CD4+ and CD8+ T cells, $CD19^{+}$ B cells, CD14+ monocytes and CD56+ NK cells in two ethnically distinct South African population groups, in the absence of HIV-1 disease. It is important to evaluate polymorphisms in chemokine ligands and receptors as they may have an impact on HIV-1 disease transmission and progression through mechanisms beyond viral entry. Given that different ethnicities exhibit remarkable differences in host genetic backgrounds, this will provide information that may assist with understanding the difference in HIV-1 control mechanisms and the variable expression levels of CXCR6 in different population groups.

7.2 Materials and Methods

7.2.1 Sample population

Refer to Chapter 2, Materials and Methods section 2.1. Briefly, black (n=17) and white (n=21) South Africans without HIV-1 were recruited and were age- and sex-matched.

7.2.2 Single nucleotide polymorphism (SNP) genotyping

Individuals were genotyped for the CXCR6 rs2234355 G>A and rs2234358 G>T SNPs as described in Chapter 2, Materials and Methods section 2.6.

7.2.3 Flow cytometry

CXCR6 expression on CD4+ and CD8+ T cells, CD19+ B cells, CD14+ monocytes and CD56+ NK cells for each individual was determined as described in Chapter 2, Materials and Methods section 2.7 and was assessed as outlined in Materials and Methods section 2.9.

7.2.4 Statistical analyses

Statistical analyses were performed as described in Chapter 2, Materials and Methods section 2.10.

7.3 Results

7.3.1 *CXCR6* rs2234355 G>A SNP and CXCR6 expression

The *CXCR6* rs2234355 SNP was only detected in the black population group (G: 70.6%, A: 29.4%, GG: 41.2%, GA: 58.8%, AA: 0%). Because no individuals were homozygous for the minor allele, possession of the wild-type genotype (GG) was compared to the heterozygous genotype (GA). We analysed the data in the black population group (n=17) as well as in the total group (n=38; all white individuals (n=21) were GG/wild-type).

Possession of the GA genotype (n=10) associated with a significantly lower frequency of CXCR6-expressing effector memory CD4+ T cells in both black individuals and in the total group (p=0.005 and p=0.026, respectively). The GA genotype also significantly associated with lower CXCR6 density on the effector memory CD4+ T cell subset in the black and total group (p=0.033 and p=0.037, respectively) and on terminally differentiated CD4+ T cells in the total group only (p=0.028). The GA genotype associated with a significantly lower frequency of CXCR6-expressing effector memory CD8+ T cells in the total group (p=0.026; p=0.001 excluding outliers). We did not see any significant associations with this SNP and CXCR6 expression when assessing total CD4+ or CD8+ T cells, emphasizing the importance of investigating smaller subsets of cells within a cell type. The GA genotype also associated with a significantly lower percentage of CXCR6-expressing B cells compared to the GG genotype in black individuals (p=0.043).

Overall, possession of the rs2234355 SNP GA genotype resulted in lower CXCR6 expression on select CD4+ and CD8+ T cell subsets as well as on B cells. A summary of findings is shown in Figure 7.1. Of note is that no p values remained significant upon correction for multiple comparisons (false discovery rate).

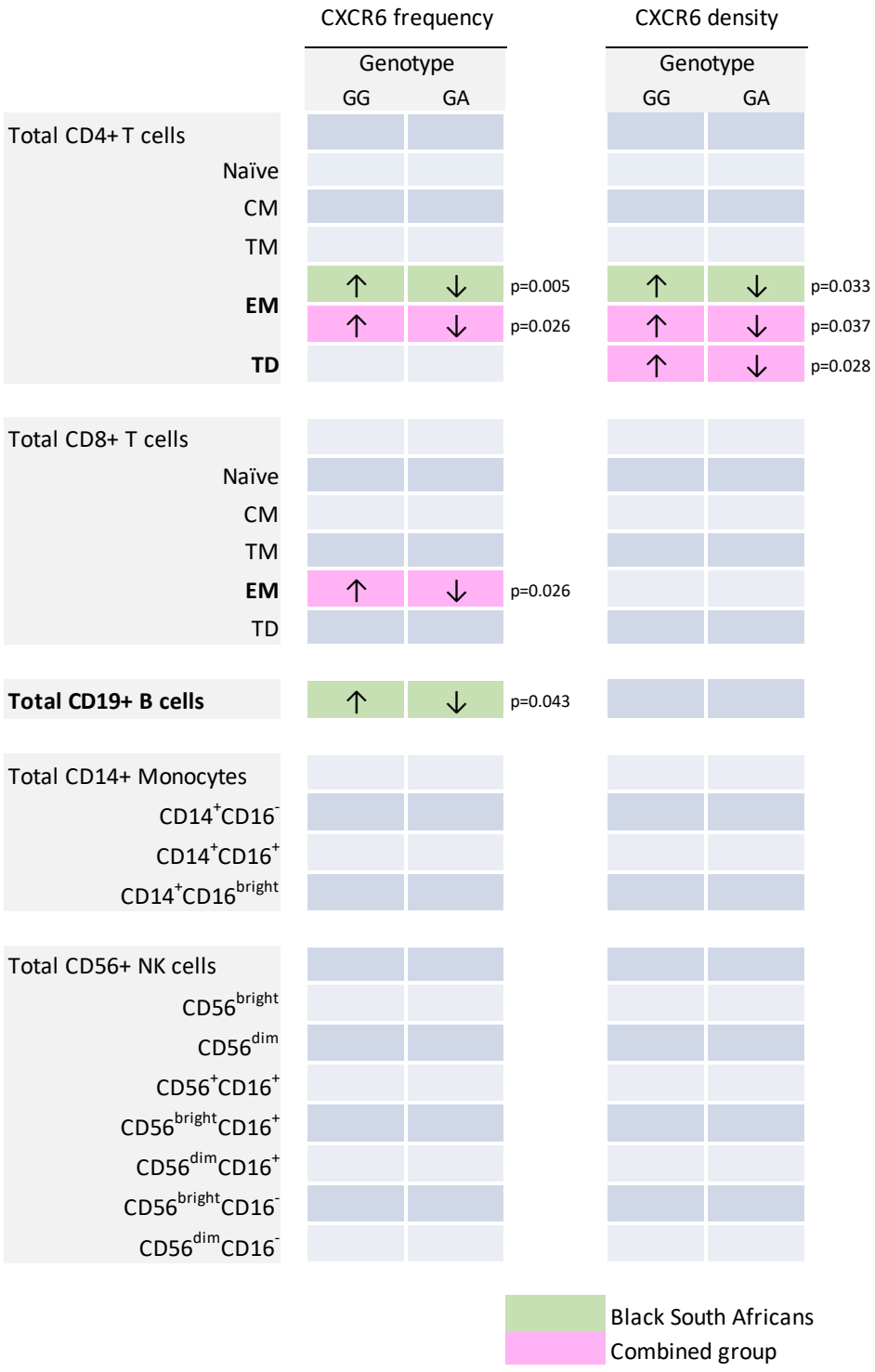


Figure 7.1 - Summary of CXCR6 expression changes associated with the rs2234355 genotype. ↑ indicates an increased expression and ↓ indicates a decreased expression associated with the respective genotype. Cell types with significant expression changes are bolded. p values for significant comparisons are shown.

7.3.2 *CXCR6* rs2234358 G>T SNP and CXCR6 expression

The *CXCR6* rs2234358 SNP was detected in both black and white individuals in our study. As reported on Ensembl's database (292), the ancestral allele is G, while the major allele in Africans is the T allele. In our study, the T allele was present at a frequency of 41.2% in black individuals and 69% in white individuals. In a previous study from our research group assessing this SNP in black and white South Africans (n=41 and n=40, respectively), the T allele was present at a frequency of 54.9% and 50% respectively, which more closely resembles data from Ensembl (135). There was a strong skewing towards possession of the TT and GT genotype compared to the GG genotype in white individuals in our study (TT: 47.6%, GT: 42.9%, GG: 0.1%), which is likely due to the small sample size in our cohort (n=17). In light of this, the T allele was used as the wild-type reference allele and TT as the dominant mode genotype (i.e., TT vs GT+GG) when analysing all data in this portion of the study.

Possession of the TT genotype associated with a significantly higher frequency of CXCR6-expressing effector memory CD4+ T cells compared to the GT genotype in white individuals (p=0.013) and compared to those with the GT+GG genotype (p=0.010). On CD8+ T cells, the rs2234358 SNP significantly associated with CXCR6 density on multiple CD8+ T cell subsets. In white individuals, the TT genotype associated with significantly higher CXCR6 density on total CD8+ T cells and transitional memory CD8+ T cells compared to individuals with the GT+GG genotype (p=0.029, p=0.005 and p=0.043, respectively). Possession of the TT genotype associated with higher density on central memory CD8+ T cells compared to the GT genotype in white individuals (p=0.010) and in the total group (p=0.018) and compared to the GT+GG genotype in white individuals (p=0.005) and in the total group (p=0.013).

The TT genotype significantly associated with a higher percentage of CXCR6-expressing B cells compared to the GG genotype in white individuals (p=0.030).

With regards to monocytes, in black individuals, the rs2234358 SNP TT genotype associated with an increased percentage of CXCR6-expressing CD14+ monocytes compared to individuals with a GT genotype (p=0.038). This was the only significant association with the rs2234358 SNP and CXCR6 expression seen in black individuals. In the total group, possession of the TT genotype associated with a significantly higher percentage of CXCR6-expressing $CD14^{+}CD16^{bright}$

monocytes compared to the GT genotype (p=0.041) and a significantly higher CXCR6 density on $CD14^{+}CD16^{-}$ monocytes compared to the GG genotype (p=0.033).

On NK cells, the GG genotype significantly associated with a lower percentage of CXCR6-expressing $CD56^{dim}CD16^{-}$ NK cells compared to both the TT genotype (p=0.030) and the GT genotype (p=0.036), only in white individuals. The TT genotype associated with significantly higher CXCR6 density on $CD56^{bright}$ NK cells compared to the GT genotype (p=0.035) and compared to the GT+GG genotype (p=0.024) and higher CXCR6 density on $CD56^{+}CD16^{+}$ NK cells compared to the GT genotype (p=0.043).

Overall, possession of the rs2234358 SNP TT genotype resulted in higher CXCR6 expression on multiple cell types, primarily in white South Africans. A summary of findings is shown in Figure 7.2. Of note is that no p values remained significant upon correction for multiple comparisons.

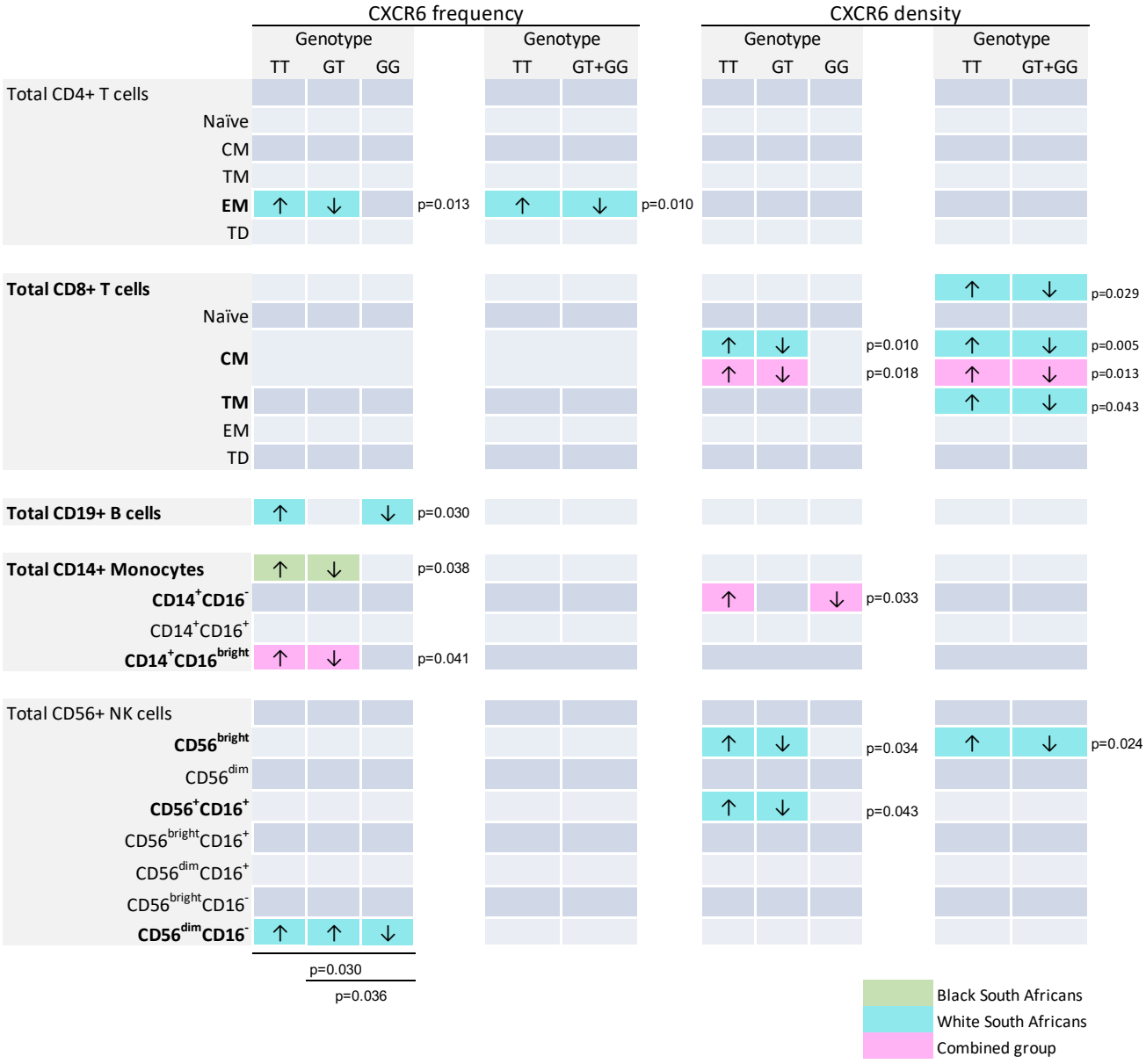


Figure 7.2 - Summary of CXCR6 expression changes associated with the rs2234358 genotype. ↑ indicates an increased expression and ↓ indicates a decreased expression associated with the respective genotype. Cell types with significant expression changes are bolded. p values for significant comparisons are shown.

7.3.3 *CXCR6* -358TT/+355GA and CXCR6 expression

Because previous work from our research group found significant associations with the combination of the possession of the rs2234355 GA genotype and the absence of the rs2234358 TT genotype (-358TT/+355GA) (135), this genotypic combination was assessed with regards to CXCR6 expression in black individuals (possession of -358TT/+355GA: n=7) and in the total group (i.e., 21 white South Africans and 10 black South Africans not having this genotypic combination, in addition to the 7 black South Africans possessing this genotypic combination). Since the rs2234355 SNP was not present in white South Africans in our study, we could not assess the effect in this population group alone.

In black South Africans and in the total group, individuals with the -358TT/+355GA genotype had a significantly lower percentage of CXCR6-expressing terminally differentiated CD4+ T cells (p=0.043 and p=0.019, respectively). In black individuals, possession of the -358TT/+355GA genotype associated with a significantly lower percentage of CXCR6-expressing CD14+ monocytes and $CD14^{+}CD16^{+}$ monocytes compared to individuals with a different genotype combination (p=0.019 and p=0.043, respectively).

With regards to CXCR6 density, in black individuals and in the total group, the -358TT/+355GA genotype combination associated with significantly lower CXCR6 density on effector memory CD4+ T cells (p=0.043 and p=0.030, respectively). In addition, in the total group, the -358TT/+355GA genotype combination associated with significantly lower CXCR6 density on terminally differentiated CD4+ T cells (p=0.013).

Overall, possession of the -358TT/+355GA genotype combination resulted in lower CXCR6 expression on select subsets of CD4+ T cells and monocytes. Of note is that no p values remained significant upon correction for multiple comparisons.

7.4 Discussion

In this study, we assessed the effects of possession of the *CXCR6* rs2234355 and rs2234358 SNPs on CXCR6 expression in black and white healthy HIV-1 uninfected South Africans. In addition, we looked at the effect of the -358TT/+355GA genotype combination on CXCR6 expression.

Because of the population-specific distribution of these SNPs, we were unable to assess all combinations in both population groups. We investigated the rs2234355 SNP in black South Africans and in the total group and found strong associations with the possession of the GA genotype and lower CXCR6 expression, both with regards to frequency and density, on subsets of CD4+ T cells, CD8+ T cells and NK cells compared to possession of the wild-type GG genotype.

A study showed that in African Americans, while time to AIDS and PCP was similar for all CXCR6 genotypes studied, individuals with the rs2234355 GG or GA genotype had a significantly lower median survival time from PCP to death and were 5.6 times more likely to die a PCP-mediated AIDS-related death compared to individuals with the AA genotype (136).

Site-directed mutagenesis of CXCR6 in Chinese hamster ovary (CHO) cells at position 3 in the *CXCR6* sequence (E3Q mutation) resulted in the loss of a negative charge and a subsequent dramatic loss of CXCR6 cell surface expression (138). When assessing intracellular and extracellular immunoreactivity and comparing to the wild-type CXCR6, the E3Q mutant exhibited less than half of the total expression, and proportionally less of the E3Q mutant construct was trafficked to the surface, with the remainder residing in a large intracellular pool (134). If the CXCR6 E3K mutant similarly struggles to traffic to the cell surface and HIV-1 utilises CXCR6 as a coreceptor for viral entry during the later stages of infection, reduced expression of CXCR6 (conferred by the CXCR6 rs2234355 A allele) would be protective (138).

In addition, patients with initial HIV-1 viral load suppression due to HAART showed a faster virologic failure in the presence of the CXCR6 rs2234355 G allele (133). Previous work from our research lab indicated that heterozygosity for the CXCR6 rs2234355 SNP (GA genotype) was likely to contribute towards viraemic control of HIV-1 (135). In our study, the rs2234355 GG genotype associated with significantly higher CXCR6 expression on multiple cell types compared to when individuals possessed the A allele, implying that the protective outcomes seen with the GA genotype may be due to the lower expression conferred by the A allele.

The CXCR6 rs2234358 SNP was the strongest signal in a study looking for genetic variants affecting HIV-1 long-term non-progressors not controlling viral load i.e., in a controller cohort excluding elite controllers who were controlling virus to very low levels (134). This association was replicated in 3 additional independent European studies and was independent of the combined

CCR2-CCR5 locus. It is important to note that all participants in these studies were white individuals of European descent. The attributable risk for the rs2234358 T allele was found to be very strong, explaining 12% of the prevention of long-term non-progression (134). For comparison, the attributable risk for *CCR5-Δ32* was 5.1% in the original cohort (327). In our study, the rs2234358 T allele associated with significantly higher CXCR6 expression on multiple cell types, demonstrating a potential biological reason for the deleterious outcomes found to be associated with this allele in HIV-1 control.

In a recent study investigating the effect of the *CXCR6* rs2234358 genotype in treatment response to direct acting antivirals in chronic hepatitis C (HCV) patients in Egypt, the GG genotype and G allele were associated with a significantly increased risk of not responding to treatment compared to individuals with the TT genotype or T allele (328). In our study, the rs2234358 TT genotype associated with increased CXCR6 expression on multiple cell types. It would be interesting to determine if this increased expression therefore is protective in HCV antiviral response, however, it is important to mention that downstream effects of SNPs that may be acting as markers may also be contributing to phenotypic outcomes.

Lastly, previous work from our research group showed a significant overrepresentation of the -358TT/+355GA genotype combination in viraemic controllers compared to healthy controls and progressors, suggesting an additive effect of the presence of the protective rs2234355 GA genotype and the absence of the deleterious rs2234358 TT genotype in natural control of HIV-1 in the presence of detectable viraemia. This prompted us to assess the association of this genotype with CXCR6 expression. The -358TT/+355GA genotype combination associated with significantly lower CXCR6 expression on multiple CD4+ T cell and monocyte subsets.

This study provides important insight into the association of two *CXCR6* SNPs (associated with HIV-1 control in the presence of viraemia) and CXCR6 expression in two distinct HIV-1 uninfected population groups. Due to the significant associations of the rs2234358 SNP and CXCR6 expression in our study being primarily seen in white individuals, more research is needed to determine if there is indeed a population-specific mechanism occurring or if a larger sample size would be helpful in assessing the true significance of this SNP in black individuals. We found that the rs2234355 SNP GA genotype resulted in lower CXCR6 expression on subsets of CD4+ T cells and CD8+ T cells and B cells, that the rs2234358 SNP T allele associated with higher CXCR6

expression on subsets of all cell types in our study and that the -358TT/+355GA genotype combination associated with significantly lower CXCR6 expression on multiple CD4+ T cell and monocyte subsets.

CHAPTER EIGHT

8. Summarising discussion and conclusions

HIV-1 remains a significant cause of morbidity and mortality, most notably in sub-Saharan Africa (8,329,330). However, research remains primarily focused on North American and European population groups (331), who have remarkably different genetic backgrounds to individuals from sub-Saharan Africa, as well as differing circulating HIV-1 subtypes. HIV-1 controllers (HICs) represent a model of HIV-1 durable control and are thus an important cohort to study. Some individuals are able to control viral replication (elite controllers and viraemic controllers) and some are able to sustain immune function in the presence of high viral loads (high viral load long-term non-progressors). Different mechanisms of control are likely to be employed by these different controller groups.

While CCR5 is one of the major coreceptors HIV-1 uses to enter cells, alternative coreceptor usage has been implicated in the less pathogenic HIV-2 and SIV strains, with the preferential usage of the CXCR6 co-receptor shown to be associated with lower pathogenicity (123,124,148). An increased expression of CXCR6 genes in transitional memory CD4+ T cells compared to central memory CD4+ T cells, and low circulating HIV-2 reservoirs mainly being distributed in transitional memory CD4+ T cells, has been described in people living with HIV-2 (147). In sooty mangabeys, a natural host for SIV infection, CXCR6 has been shown to be restricted to CD4+ effector memory T cells and was expressed by a separate sub-population to cells expressing CCR5 (123). In sooty mangabeys infected with SIV, central memory CD4+ T cell reservoirs are limited, potentially due to low CCR5 coreceptor expression in this cell subset (156). The lack of infection of central memory CD4+ T cells seems to be a factor in low pathogenicity models of both HIV and SIV (156,161).

The discernable differences in the pathogenicity of HIV-1 and SIV allows for a unique opportunity to explore protective viral and host immune mechanisms that may be contributing to viral control. Since high viral load long-term non-progressors exhibit a similar phenotype to SIV-infected sooty mangabeys, and CXCR6 polymorphisms have been associated with viraemic HIV-1 control (134,135), CXCR6 may be of importance in this unique group of controllers. The potential interplay of CCR5 and CXCR6 therefore requires further study.

This study was thus undertaken to investigate genetic variants in CCR5 and HIV-1 control in people living with HIV-1 (controllers and progressors), and to assess CCR5 and CXCR6 constitutive expression and potential association with genetic polymorphisms in HIV-1 uninfected individuals using various methodologies, including single nucleotide polymorphism (SNP) genotyping, Sanger sequencing and flow cytometry. The effect of ethnicity, cell activation, sex and age on these findings was also described.

8.1 *CCR5* polymorphisms associating with HIV-1 natural control in black South Africans

When characterising polymorphisms in the *cis*-regulatory regions of *CCR5* in black South African HIV-1 controllers and progressors (Chapter 3), our results reproduced other studies in showing that the *CCR5* HHE haplotype is deleterious with respect to HIV-1 disease progression, and the HHA haplotype and HHA/HHC genotype associated with protection from HIV-1 disease progression (112,116,332,333). We characterized novel haplotypes in the 3'UTR as well as haplotypes spanning the *CCR5* 5'UTR and 3'UTR. The variant that most strongly associated with HIV-1 progression was the *CCR5* +2919 T>G SNP, both heterozygosity and in the dominant mode. To our knowledge, no other studies have associated this SNP with increased risk of HIV-1 disease acquisition or more rapid progression of HIV-1, emphasizing the importance of population-specific studies (334). Our results suggest that two *CCR5* promoter SNPs (-2459 G>A and -2135 T>C) and one *CCR5* 3'UTR SNP (+2919 T>G) may be key functional variants with regards to HIV-1 control in black South Africans.

8.2 Differences in proportions of T cells in black and white South Africans

Using flow cytometry, proportions of the different CD4+ and CD8+ T cell subsets (naïve, central memory, transitional memory, effector memory and terminally differentiated) were assessed in the absence of HIV-1 infection in black and white South Africans. Black individuals had a significantly higher proportion of terminally differentiated CD8+ T cells compared to white individuals. An 'immune risk phenotype' has been identified which includes expansion of terminally differentiated CD8+ T cells, an inverted CD4+/CD8+ T cell ratio and HCMV positivity, and is a strong predictor of mortality (335–337). The presence of increased terminally differentiated T cells during HIV-1 disease is often suggestive of exhaustion, with a resulting dysregulation of T cell homeostasis and function and a subsequent rapid progression of HIV disease (238).

Terminal differentiation of T cells has been strongly associated with HCMV infection and was found to be increased in individuals living with HIV-1 on ART (338). People living with HIV-1 are almost universally coinfected with HCMV, with both HCMV and HIV-1 associating with inflammation and aging (335,339). Whether HCMV accelerates HIV pathogenesis or acts as an opportunistic pathogen as a result of the general impairment in cellular immune responsiveness caused by HIV is not yet fully clear (340). While various studies have implicated HCMV as a contributor to the pathogenesis of AIDS (340–343), its direct effect is difficult to determine since most adults living with HIV-1 are infected with CMV prior to acquisition of HIV-1.

Ethnicity has previously been linked to HCMV prevalence, with non-white race groups exhibiting a higher occurrence (344–346). Previous work from our research group (Shalekoff, unpublished data), demonstrated that significantly more black South Africans were seropositive for HCMV compared to white individuals. It is unclear whether the overburden of HCMV in non-white population groups is due to socioeconomic factors or host genetics.

Regardless, based on ethnicity alone, an increased pool of terminally differentiated CD8+ T cells in black compared to white South Africans is suggestive of a baseline difference in potential immune regulation and control of disease.

8.3 CCR5 and CXCR6 constitutive expression differences between black and white South Africans

Since HIV-1 infection modulates the expression of chemokines (347–349), constitutive expression levels of CCR5 and CXCR6 were assessed in healthy HIV-1 uninfected black and white South Africans to determine population-specific differences (Chapter 4). CCR5 and CXCR6 frequency (percentage-expressing cells) and density (number of molecules per cell) were measured on CD4+ and CD8+ T cells, B cells, monocytes and natural killer (NK) cells using flow cytometry.

8.3.1 CCR5 expression differences

Systemic immune activation is a feature of HIV-1 infection and increased T cell activation is an independent predictor of HIV-1 progression (191,192). In addition to being an integral molecule in the entry of R5 HIV-1 viruses into target cells, CCR5 is also a co-activation molecule (190). In individuals where HAART was either started or stopped, the subsequent change in immune

activation did not alter CCR5 density, suggesting that baseline levels of CCR5 expression affect the extent of lymphocyte activation (190).

Data from this study suggests that there are significant population-specific differences in CCR5 expression, with black South Africans predominantly exhibiting a lower expression level of CCR5 compared to white South Africans. Strongly significant findings included a lower percentage of CCR5-expressing $CD14^{+}CD16^{bright}$ non-classical monocytes and $CD56^{dim}CD16^{-}$ NK cells, and lower CCR5 density on total CD8+ T cells and central memory CD4+ T cells, in black compared to white individuals.

8.3.1.1 $CD14^{+}CD16^{bright}$ monocytes

HIV-1 has been shown to infect monocytes, particularly the CD16+ intermediate and $CD16^{bright}$ non-classical monocytes (350,351). Monocytes and macrophages critically influence HIV transmission and viral spread occurring early in infection and chronically infected macrophages serve as a reservoir for HIV-1 (220,352–354). CD16 + monocytes dominate in infectious and inflammatory conditions like HIV (355,356) and are preferentially infected and retrieved from blood compared to classical monocytes, even when an individual has sustained viral suppression (350). Further demonstrating the role of CD16+ monocytes as HIV-1 viral reservoirs, they have been shown to contain HIV-1 viral variants genetically different from sequences found in resting CD4+ T cells (357).

Tissue macrophages are a major target for HIV-1. While monocyte-derived macrophages (MDM) are not killed by HIV-1, they have been shown to produce virus in cultures for as long as several weeks (354). Therefore, MDM have the potential to act as long-lived reservoirs for HIV-1, aiding in the spread of virus to other tissues (221,353). A direct association has been found between levels of CCR5 and the differentiation of monocytes to macrophages, and CCR5 expression associated with monocyte resistance and macrophage susceptibility to HIV-1 infection, providing direct evidence that CCR5 functions as a coreceptor for HIV-1 infection of primary macrophages (354).

Black individuals in our study had a lower percentage of CCR5-expressing $CD14^{+}CD16^{bright}$ monocytes compared to white individuals. These cells are responsible for CD4+ T cell proliferation and stimulation and stimulate the production of IL-4 by CD4+ T cells (356,358). Additionally, it was shown that $CD14^{+}CD16^{bright}$ monocytes are involved in the overproduction of TNFα in ART-

naïve, HIV-infected individuals without viraemic suppression, suggesting that this cell subset might be a major contributor to the immune hyperactivation of the disease (359,360).

8.3.1.2 $CD56^{dim}CD16^{-}$ NK cells

Traditionally, NK cells have been assessed as $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ populations, with the former being responsible for cytokine production, preferentially concentrating in secondary lymphoid tissues, and the latter demonstrating enhanced cytotoxicity and occupying peripheral blood, lungs and areas of inflammation (361–365). $CD56^{dim}CD16^{-}$ NK cells are likely to be a heterogenous population comprising both maturing and target cell-activated cells (366). The $CD56^{dim}CD16^{-}$ subset was found to be the dominant NK cell population in melanomas and to express the largest repertoire of chemokine receptors, including CCR5, implying that this subset is highly proficient at recruitment into inflamed tissues (367). Ethnic variation of CCR5 expression on this subset may thus be indicative of differential trafficking and recruitment depending on the population studied.

8.3.1.3 CD8+ T cells

HIV-1 controllers are more likely to sustain long-term memory and effector potential CD8+ T cell responses compared to progressors, allowing for an increased ability to kill infected cells prior to progeny virion production (306,307). Effector memory CD8+ T cells are characterized by a high cytotoxic capacity (308). As HIV/SIV disease progresses, HIV-specific CD8+ T cell cytotoxicity decreases considerably until cells no longer exert a suitable antiviral response (309,310).

In a study conducted on healthy individuals, a correlation was found between the chemotactic response of peripheral blood CD8+ T cell to CCL5 and the level of surface CCR5 expression (368). While CCR5 density on T cells was found to remain constant over time for a given individual, inter-individual variation of CCR5 density was found to vary drastically, which may be an important personal determinant of T cell migration in multiple biological situations where CCR5-binding chemokines play a role (368).

8.3.1.4 Central memory CD4+ T cells

The finding of lower CCR5 density on central memory CD4+ T cells in black individuals in our study is noteworthy, since multiple studies have indicated that central memory CD4+ T cells are

the main T cell subset that correlates to the loss or preservation of CD4 cells in HIV or SIV disease (150,158,369). CCR5 density on CD4+ T cells is an important factor in HIV-1 disease progression, with high CCR5 levels on central memory CD4+ T cells in acute HIV infection associating with rapid disease progression (370). CCR5 density was found to correlate with, and be predictive of, the immune activation levels of people living with HIV-1, independently of HIV-1 viral load (190). Thus, low CCR5 expression on central memory CD4+ T cells is protective, resulting in a reduction of direct viral infection of CD4+ T cells and aiding in the preservation of CD4+ T cell homeostasis (370).

With constitutively lower CCR5 expression on non-classical monocytes, $CD56^{dim}CD16^{-}$ NK cells, CD8+ T cells and central memory CD4+ T cells in black compared to white South Africans, there is likely to be baseline differential immune control of disease exhibited by these ethnic groups.

8.3.1.5 CCR5-CXCR4 co-receptor switching and HIV-1 pathogenesis

While CXCR4 usage tends to be limited to subtype B strains, being less common in the subtype C-driven HIV-1 transmissions in sub-Saharan Africa (34), the increase in pathogenicity after viruses switch receptor usage to CXCR4 is an important factor with regards to CD4+ T cell decline and HIV-1 disease progression (31,33). CCR5 density correlates with the immune activation levels of people living with HIV-1, and CD4+ T cell activation has been shown to predict the emergence of viral strains that preferentially use CXCR4 (190).

Naïve and central memory CD4+ T cells tend to express the highest levels of CXCR4 (371). While the significance of this is not yet elucidated, it is interesting then that black individuals in our study constitutively exhibited a significantly lower CCR5 density on these subsets compared to white individuals. Since T cell activation has been shown to precede and independently predict X4-tropism switching (372), the potentially lower activation state of these subsets in black individuals may be protective with regards to HIV-1 pathogenesis, in addition to the protective effects of lower CCR5 density with regards to HIV-1 cell entry and CD4+ T cell infection (98,104,370,373).

It is not known whether the major protective effect of low CCR5 expression is due to lowered immune activation or the direct inhibition of HIV-1 cellular entry, however, it is likely that both of these mechanisms contribute to HIV-1 control. Further study using cloning, flow cytometry and

viral vector-based assays will be helpful in determining the effect of low expression levels of CCR5 on immune activation and/or viral entry.

8.3.1.6 CCR5-based cure strategies

Subsequent to a stem cell transplant from a homozygous *CCR5Δ32* donor, the 'Berlin patient', Timothy Ray Brown, was functionally cured of HIV-1; his HIV-1 viral load decreased to undetectable limits (374,375). This case inspired multiple investigations into CCR5 as a target for a functional HIV-1 cure. Researchers have examined the inhibition of extracellular CCR5 through the use of small molecule inhibitors or monoclonal antibodies, in addition to exploring the impact of preventing CCR5 expression through various gene editing techniques such as RNA interference, Transcription Activator-Like Effector Nucleases (TALENS), Zinc Finger Nucleases (ZFN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), on HIV-1 progression and acquisition (90). Leronlimab is a monoclonal antibody directed towards CCR5 that binds CCR5 with high affinity, inhibiting HIV-1 entry into CD4+ T cells (376,377). With different ethnicities exhibiting varied levels of constitutive CCR5 expression, such as in this study, the design of these CCR5-based cure strategies will need to take ethnicity into account when determining the effectiveness of these interventions.

8.3.2 CXCR6 expression differences

CXCR6 expression only differed between population groups with regards to percentage of CXCR6-expressing cells, not CXCR6 density, with black individuals having a lower percentage of CXCR6-expressing CD8+ T cell subsets (naïve and effector memory) and a higher percentage of CXCR6-expressing $CD14^{+}CD16^{+}$ monocytes compared to white individuals.

8.3.2.1 Naïve and effector memory CD8+ T cells

During pathogenic lentiviral infection, an increased turnover of naïve and memory T cells has been noted (378–380). In chronic HIV-1 infection, memory CD4+ but not memory CD8+ T cells have been demonstrated to decrease in number, while both naïve CD4+ and naïve CD8+ T cells are depleted, leading to the conclusion that naïve T-cell depletion is one of the major hallmarks of HIV infection (378,381,382).

The CD8+ T cell response is a central mechanism of viral control present in the majority of HIV-1 controllers, and the phenotype of these cells, with regards to effector and memory phenotypes and in level of activation, may therefore differentially impact on viral control (383). *In vitro,* in HIV-controllers, the effector memory and terminal effector subpopulations of CD8+ T cells possessed a high inhibitory potential that suppressed HIV-1 infection, and these subpopulations responded more rapidly to infection (384). In an SIV model, similar results were described, where vaccination induced high numbers of effector memory CD8+ T cells, resulting in the establishment of a controller phenotype (383,385).

CXCR6-expressing CD8+ T cells have been implicated in anti-tumour activity (386–388). CXCR6 has also been shown to be crucial in the development and preservation of protective memory CD8+ T cells in the liver (389). CXCR6 is crucial for tissue homing T cells that get triggered via locally expressed CXCL16, the ligand for CXCR6 (390–392). Thus, the ethnicity-based variation of CXCR6 expression levels on these cell subsets seen in our study is likely to result in a differential control of not only HIV-1, but other inflammatory conditions.

8.3.2.2 $CD14^{+}CD16^{+}$ monocytes

As mentioned in Section 8.3.1.1, HIV-1 preferentially infects CD16+ intermediate and $CD16^{bright}$ non-classical monocytes over CD16- monocytes, and CD16+ monocytes act as a viral reservoir for HIV-1 (350,351,357). $CD14^{+}CD16^{+}$ monocytes express the highest levels of antigen presentation-related molecules compared to the other monocyte subsets (359,393,394) and express more CCR5 than classical monocytes, likely accounting for their high susceptibility to HIV-1 infection (350,395,396). $CD14^{+}CD16^{+}$ monocytes populations are expanded in individuals with systemic infections, implying that this subset is integral in the rapid defense against pathogens (359,397,398).

Since black individuals have a higher percentage of CXCR6-expressing $CD14^{+}CD16^{+}$ monocytes compared to white individuals in our study, a lower potential for HIV-1 reservoirs in this cell subset for black individuals might be plausible if diverting HIV-1 from CCR5-expressing $CD14^{+}CD16^{+}$ monocytes, especially since CCR5 and CXCR6 expression on monocytes did not correlate in our study.

8.4 Relationship between CCR5 and CXCR6

To learn more about the relationship between CCR5 and CXCR6 in the same individuals and on the same cell type, we evaluated the ratio of CCR5 to CXCR6 expression (both frequency and density) and correlated the expression levels of these two coreceptors on the different cell types. It is important to note that due to the limitation of needing to have PE-labeled antibodies to both CCR5 and CXCR6 in order to quantitate the number of molecules of CCR5/CXCR6 per cell, CCR5 and CXCR6 could not be assessed on the same flow cytometry panel. We still, however, found it to be informative to assess the ratio and correlation of expression levels of these two chemokine receptors in order to gain more understanding with regards to the relationship between CCR5 and CXCR6 expression.

8.4.1 CCR5 and CXCR6 expression ratios

The ratio of CCR5 to CXCR6 expression (frequency and density) on each cell subset studied was calculated for every individual and the medians of the population groups were compared.

The ratio of CCR5 to CXCR6 expression differed most strongly between population groups on effector memory CD8+ T cells (black South Africans had a lower median ratio of CCR5 to CXCR6 density compared to white South Africans), $CD14^{+}CD16^{bright}$ monocytes (black South Africans had a lower median ratio of CCR5-expressing to CXCR6-expressing $CD14^{+}CD16^{bright}$ monocytes compared to white South Africans) and $CD56^{bright}CD16^{+}$ NK cells (black South Africans had a higher median ratio of CCR5-expressing to CXCR6-expressing $CD56^{bright}CD16^{+}$ NK cells compared to white South Africans).

Since ratios were calculated with CXCR6 as the denominator, a ratio of >1 indicates higher CCR5 expression compared to CXCR6 expression. For example, in this study, black South Africans had CCR5 and CXCR6 expression levels on effector memory CD4+ T cells that exhibit less of a difference while white South Africans have a larger difference between CCR5 and CXCR6 expression on these cells (with higher CCR5 expression compared to CXCR6 expression). If more CXCR6 expression is preferable with regards to HIV-control (as has been seen in SIVsmm and HIV-2), a lower median ratio would be considered more protective compared to a higher ratio, as is seen in black individuals in this example, especially if the ratio is <1, signifying a higher CXCR6 expression compared to CCR5 expression on that cell type. To our knowledge, there are no other

analyses of the ratio of CCR5 to CXCR6 expression in the literature, thus making it difficult to compare and contextualize these findings.

8.4.2 CCR5 and CXCR6 expression correlations

CCR5 and CXCR6 expression exhibited strong positive correlations on most CD4+ T and CD8+ T cell subsets, B cells and two NK cell subsets ($CD56^{+}CD16^{+}$ and $CD56^{bright}CD16^{+}$). It is interesting that both the frequency and density of CCR5 expression significantly positively correlated with CXCR6 expression on multiple subsets of CD4+ and CD8+ T cells. This provides insight into the possible relationship between the two receptors. CC and CXC chemokines have previously been shown to synergise, increasing leukocyte recruitment to areas of inflammation (214). Synergistic interactions between chemokines may contribute to the enhancement and modification of inflammatory responses (215). Since T cells are essential to immune responses, it is likely that differing expression of CCR5 and CXCR6 on these subsets in the different population groups may impact various disease outcomes depending on ethnicity. Studies specifically designed to assess the complex relationship between these two important coreceptors are needed.

8.5 Activation and effect on CCR5 and CXCR6 expression

As already mentioned, one of the greatest predictors of HIV-1 progression is hyperactivation of T cells (191,192). It is widely accepted that cell activation results in an increase in CCR5 expression (95,205,228). Activated CD4+ cells, defined by expression of HLA-DR, express CCR5 at higher levels than non-activated or HLA-DR- CD4+ cells (95,187,317). CXCR6 expression has been observed on both activated and unstimulated CD8+ T cells, activated and naive CD4+ T cells, NK cells, natural killer T (NKT) cells and CD19+ B cells (159,193,399) and has been shown to be upregulated following T cell activation (236,400,401).

CCR5 and CXCR6 expression was evaluated to determine if there was an increase in expression on activated cells in our population groups, using HLA-DR as a marker for activation. The main findings indicated that black individuals had significantly lower CCR5 density on HLA-DR+ CD4+ T cells and significantly lower CXCR6 density on HLA-DR+ monocytes compared to white individuals. While lower CCR5 density is known to be protective in the context of HIV-1 acquisition and progression (98,104,105,317,373), the implications of lower CXCR6 density on activated monocytes is not yet clear. CXCR6 has been shown to promote monocyte infiltration and

studies have shown that knocking out CXCR6 results in lower inflammation and lower fibrosis in organs like the kidney, liver and heart in multiple disease states (359,402,403), therefore it may be hypothesized that a lower CXCR6 density on activated monocytes is protective against inflammation induced fibrosis.

8.6 Impact of sex and age on CCR5 and CXCR6 expression

Since a variety of factors can affect the expression levels of chemokines and their associated receptors, including sex and age, we considered the effect of these variables on CCR5 and CXCR6 expression (Chapter 5). Many studies fail to address sex and age differences in disease acquisition and progression.

Sex differences have been shown to exert a sizeable effect on outcomes of HIV-1 disease (251–253). Females living with HIV-1 have been shown to have higher levels of innate and adaptive immune responses compared to males (254). In macaques infected with SIV-1, central memory CD4+ T cells from females were found to differentially regulate a significantly larger number of genes at day 4 post-infection when compared to males, with female macaques having significantly higher levels of the CCL2, I-TAC and MIF cytokines, suggesting that early innate cytokine responses to SIV lean towards a more pro-inflammatory phenotype in female macaques (255). In mice thymocytes, age-associated changes in the expression of genes involved in T cell receptor signaling, antigen presentation and lymphocyte development and function were identified, including an increased expression of CXCR4 and CXCR6 with age, and a decrease in CCL25 (263).

In our study, both sex and age affected CCR5 and CXCR6 expression, however results varied widely across the two population groups studied. Results were population-specific, with sex only having an effect on CXCR6 expression in white individuals (with predominantly increased CXCR6 expression in males compared to females). The strongest findings were a higher percentage of CCR5-expressing central memory CD8+ T cells (in black individuals) and a higher percentage of CCR5-expressing $CD56^{bright}CD16^{-}$ NK cells in males compared to females (in white individuals).

Age also primarily associated with CCR5 and CXCR6 expression in white individuals. Older age associated with lower CCR5 and CXCR6 expression on NK cells, and a higher CXCR6 frequency but lower CXCR6 density on naïve CD8+ T cells. Older age associated with a higher CCR5 density but a lower CXCR6 density on CD8+ T cells. Whereas age tended to associate with higher CCR5

expression (frequency and density) on monocytes across population groups, increasing age associated with lower CXCR6 expression (frequency and density) on these cells. A consistent finding across chemokine receptors was older age strongly associating with lower CCR5 and CXCR6 expression (frequency and density) on multiple NK cell subsets.

Whether these population-specific sex and age differences in CCR5 and CXCR6 expression translates into differential HIV-1 control between the two population groups requires further investigation, but this study highlights the importance of taking sex and age into consideration in studies involving disease and disease outcomes.

Overall, we found significant population-specific differences in expression levels of both CCR5 and CXCR6, found multiple associations with cell activation and CCR5 and CXCR6 expression and found that CCR5 and CXCR6 expression significantly correlated on multiple cell subsets. CD8+ T cells and NK cells showed the strongest ethnic differences with regards to CCR5 and CXCR6 expression, therefore further study on these cell types is recommended to determine the extent to which this is environmentally driven and influenced by host genetics and what the implications of these findings are on immunological functioning.

8.7 *CCR5* and *CXCR6* gene variants and expression

Having established that select *CCR5* and *CXCR6* variants associated with natural control of HIV-1 (115,134–136,300,333), their role in expression of these receptors was evaluated using the data we generated for healthy controls.

8.7.1 *CCR5* variants

Four *CCR5* variants were selected for evaluation based on their association with HIV-1 control (Chapter 6). The rs553615728 -4223 C>T SNP disrupts a cytidine phosphate guanidine (CpG) dinucleotide in the *cis*-region of *CCR5,* CpG-41, a binding site where DNA methylation occurs, and is uniquely found in individuals from southern Africa (115). The -4223 C>T SNP associated with protection from HIV-1 acquisition in black South Africans in a previous study, however, associations did not reach statistical significance (115). We could not assess the relationship between the -4223 SNP and CCR5 expression due to the low frequency of this SNP in our sample population.

The *CCR5* promoter rs1799987 −2459 G>A SNP has been linked to differences in CCR5 expression levels on CD14+ monocytes (178) and has been associated with the rate of progression to AIDS (117–119). The -2459 G>A minor allele is present in the *CCR5* HHE, HHF*1, HHF*2, HHG*1 and HHG*2 promoter haplotypes. Our previous work also indicated that this variant associated with HIV-1 control (Chapter 3). We found that the -2459 G>A SNP had multiple associations with CCR5 expression. However, no results remained significant after correction for multiple comparisons. The GG genotype tended to associate with an increased CCR5 expression compared to the GA and AA genotype, except on naïve CD8+ T cells (frequency), $CD14^{+}CD16^{bright}$ monocytes (density) and $CD56^{dim}CD16^{-}$ NK cells (frequency), which is unexpected because individuals with the GG genotype have been shown to exhibit slower disease progression than those with the AA genotype (117–119). Again, results varied across population groups.

The 3'UTR rs746492 +2919 T>G SNP significantly associated with HIV-1 disease progression in a black South African population (Chapter 3), but has not, to our knowledge, been assessed with regards to HIV-1 control in other population groups (334). When evaluating the effect of this variant on CCR5 expression, the +2919 TG genotype associated with a significantly higher percentage of CCR5-expressing total CD8+ T cells, transitional memory and terminally differentiated CD8+ T cells compared to the GG genotype in the total combined group, and the +2919 GG genotype associated with a significantly lower percentage of CCR5-expressing B cells compared to the TT and TG+TT genotypes in white South Africans. The +2919 TG and TG+TT genotypes associated with significantly higher CCR5 density on all CD8+ T cell subsets except naïve CD8+ T cells compared to the GG genotype in white South Africans and the total combined group.

With the +2919 TG genotype associating with higher CCR5 expression (both with regards to frequency and density) on subsets of CD8+ T cells (only in white individuals and in the total group), this finding potentially offers a functional reason for the significant overrepresentation of the TG genotype in HIV-1 progressors compared to controllers reported in this study (Chapter 3). Since CD8+ T cells are not known to be reservoirs for HIV-1, the deleterious consequences of higher CCR5 expression on these cells could be due to the fact that an increase in CD8+ T cell activation and exhaustion has been shown to result in inefficient viral control (404).

Many studies have demonstrated the relevance of CCR5 receptor density in the context of HIV-1 infection and that the amount of CCR5 expressed on the cell surface directly influences an individual's susceptibility to HIV-1 (189,315,316). It is important to note, however, that in our study, the overrepresentation of the TG genotype in HIV-1 progressors was in black South African individuals, and the genotype's significant association with higher CCR5 expression was only seen in white individuals and in the total group. This could be due to the small sample size of black individuals, although the strength of the findings in the white population group even with low numbers is interesting. The frequency of the TG genotype did not differ significantly between black and white individuals in our study, suggesting that other factors are likely to be involved in the significantly lower CCR5 density on CD8+ T cells seen in black individuals compared to white individuals in this study (Chapter 4).

The fourth SNP assessed was the rs1015164 G>A polymorphism that marks expression of the antisense long non-coding RNA (lncNRA) CCR5AS, a variant that genome wide association studies (GWAS) showed had strong associations with HIV-1 outcomes (121). In our cohort, this variant did not associate with CCR5 expression in either population group. It is unclear whether this is due to the small sample size of our cohort or whether this SNP is marking other SNPs that are contributing functionally to HIV-1 control. For instance, the authors mention that since the rs1015164 SNP is intergenic and not predicted to be located in a transcription factor binding site, it is unlikely to be directly responsible for differential CCR5AS expression levels seen in their study (121). The CCR5AS intronic rs2027820 variant, however, which is in almost perfect LD with rs1015164 (r^2=0.9–1), has been shown to regulate expression of CCR5AS through the differential binding of activating transcription factor 1 (ATF1) (121).

Our study and the study that showed an association with rs1015164 and CCR5 expression levels exhibit multiple differences. In the study by Kulkarni et al., (2019), the effect of the various genotypes on expression was assessed using qPCR to measure mRNA expression levels, and the study participants were of Japanese descent infected with HIV-1 subtype B, consisting of 92% male individuals. Additionally, they did not directly assess the effect of this SNP on CCR5 levels, but evaluated its association with CCR5AS expression levels (121).

8.7.2 *CXCR6* variants

In Chapter 7, we explored two *CXCR6* genetic variants (rs2234355 G>A and rs2234358 G>T) and their association with CXCR6 expression on CD4+ and CD8+ T cells, B cells, monocytes and NK cells in healthy individuals. The rs2234358 SNP has been associated with HIV-1 control in viraemic controllers (VCs), independent of the CCR2-CCR5 locus (405), and the rs2234358 TT genotype was found to be significantly underrepresented in VCs compared to black South African healthy controls and progressors (135). In our study, while no associations remained significant after correction for multiple comparisons, possession of the rs2234355 SNP GA genotype associated with lower CXCR6 expression on select CD4+ and CD8+ T cell subsets as well as on B cells, and possession of the rs2234358 SNP TT genotype associated with higher CXCR6 expression on multiple cell types, primarily in white South Africans.

When examining genotype representation in HIV-1 controllers and progressors, an additive protective effect of the absence of the 'deleterious' rs2234358 TT genotype and the presence of 'advantageous' rs2234355 heterozygosity (-358TT/+355GA) was found (135). In our study, possession of the -358TT/+355GA genotype combination associated with lower CXCR6 expression on select subsets of CD4+ T cells and monocytes, seemingly suggesting that a lower expression of CXCR6 on these subsets may be beneficial in HIV-1 control, such as is the case with CCR5. It is already well established that higher CCR5 expression is deleterious with regards to HIV-1 acquisition and progression (96,97,317), however, it is not yet determined whether high CXCR6 expression is also deleterious in the context of HIV-1. Studies conducted using larger cohorts will help elucidate the true role of these two variants on CXCR6 expression and ultimately, HIV-1 control.

8.8 Study limitations

The limitations of our study include the small sample sizes present in each ethnic group studied. In addition, analyses on peripheral blood may not be reflective of what is happening in the germinal centers and lymphoid tissue.

The way that we assessed the effect of individual SNPs on expression levels does not take into account other SNPs in the gene or other distinct genes that may be marked by the SNPs we assessed.

Therefore, we cannot confidently say that the results we see are indeed due to the specific SNP studied.

We did not conduct correction for multiple comparisons on the expression data in our study, which would likely result in a loss of many significant findings - due to the exploratory nature of this study, we found it informative to include significant results without correcting for multiple comparisons. We did, however, correct for multiple comparisons for the genetic association studies, since statistical analysis of genetic association data needs to estimate many effects and test many hypotheses, resulting in false positives, or the Type I error rate, increasing with each additional test (406–408). Lastly, CCR5 and CXCR6 were not assessed in the same flow cytometry panel because we wanted to determine the density of these receptors on the cell types studied, which required separate panels.

8.9 Conclusion and future recommendations

While we are aware of the study limitations, novel insight was gained with regards to CCR5 and CXCR6 in a South African context. We characterized polymorphisms (SNPs and indels) and intragenic haplotypes within the *cis*-regulatory regions of *CCR5* in black South African HIV-1 controllers and progressors, identified previously unreported polymorphisms and haplotypes and expanded upon previously defined haplotypes within the *CCR5* gene, in addition to associating variants with HIV-1 control. This study also provides further understanding into constitutive CCR5 and CXCR6 expression levels on CD4+ and CD8+ T cells, B cells, monocytes and NK cells in black and white South Africans without HIV-1, and the potential associations of select genetic variants with expression. In our study, we illustrated that CXCR6 expression tends to follow a similar pattern of expression to CCR5, positively correlating with CCR5 expression on a number of cell subsets.

Although our sample sizes are small and findings needs to be validated in larger cohorts, this work provides important baseline reference data and highlights the necessity of ethnicity-, age- and sex-specific studies. This study clearly shows that these variables have an effect on constitutive expression of CCR5 and CXCR6, which would have important implications for individuals in a disease state. It is therefore our recommendation to consider ethnic and sex differences in the design of studies assessing HIV-1 infection, as well as in other disease research. CD8+ T cells and NK

cells were found to exhibit the greatest inter-population variability with regards to expression, SNP associations and activation effects. Future work including larger sample sizes and the use of functional assays to assess the direct link between genetic variants and expression would be helpful to determine population-specific differences and implications on HIV-1 control. Assessing CCR5 and CXCR6 expression in the same flow cytometry panel to determine the relationship between these two coreceptors on the same cells would additionally provide useful information.

While CCR5 and CXCR6 act as coreceptors for HIV-1 and SIV, they are also both important molecules with regards to chemotaxis and are involved in both immune and inflammatory responses. Ethnic differences with regards to expression of these two molecules thus gives important insight into potential population driven immune differences, which is applicable for HIV-1 as well as other diseases.

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APPENDIX A: Ethical Clearance

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Professor CT Tiemessen, et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190995

NAME: (Principal Investigator)	Professor CT Tiemessen, et al
DEPARTMENT:	National Institute for Communicable Diseases Centre for HIV and Sexually-Transmitted Infections Sandringham
PROJECT TITLE:	HIV-1 positive South African Elite and Long-term Controllers: viral and host targets for functional cure strategies
DATE CONSIDERED:	Ad hoc
DECISION:	Aproved unconditionally
CONDITIONS:	Renewal of M140926
SUPERVISOR:	Not applicable
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2019/10/04

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

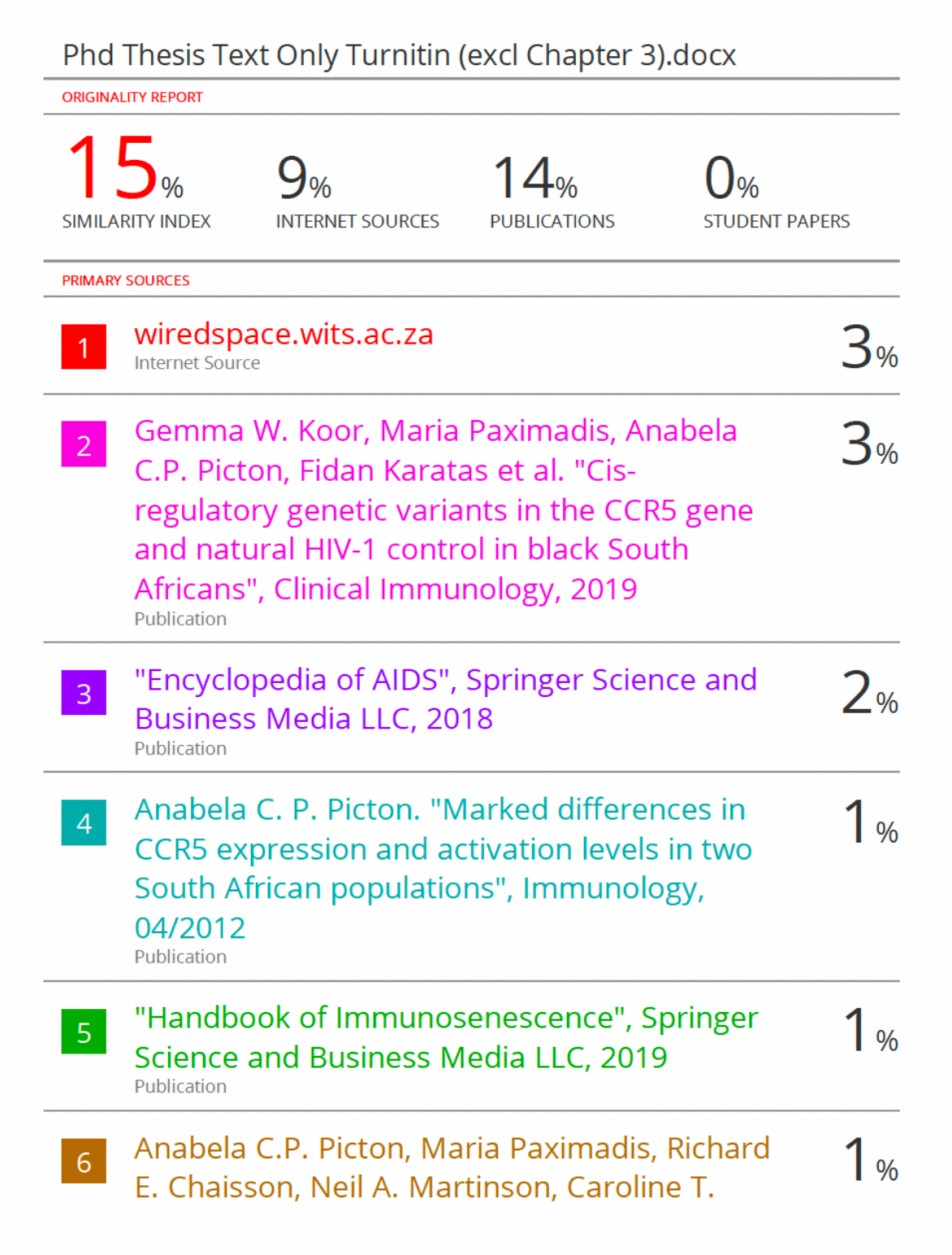
DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. I **agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **September** and will therefore reports and re-certification will be due early in the month of **September** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature — Date: 5 October 2019

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES

APPENDIX B: Turnitin originality report



Phd Thesis Text Only Turnitin (excl Chapter 3).docx

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