THE INFLUENCE OF HELMINTHS ON IMMUNE RESPONSES TO HIV

by

Zilungile L. Mkhize-Kwitshana

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

in the

School of Pathology and Laboratory Medicine:
Microbiology Department
Discipline: Immunology
N.R.Mandela School of Medicine

University of KwaZulu-Natal
Durban

2009

DEDICATED TO MY LATE PARENTS

NOMUSA & SOBUTHONGO MKHIZE

Declaration

This study represents original work by the author and has not been submitted in any form to another University. Where assistance was received and use of the work of others was made it was duly acknowledged in the text.

The work described in this dissertation was carried out in the Immunology Group, Division of Molecular Biology and Human Genetics Biochemistry, Department of Biomedical Sciences, Faculty of Health Sciences at the Stellenbosch University, Western Cape Province, South Africa under the supervision of Prof. G. Walzl, (Head-Immunology Group) and Prof. M Taylor (Department of Public Health Medicine, University of KwaZulu-Natal) as co-supervisor. Statistical assistance was offered by the South African Medical Research Council's Biostatistician, Dr Lize van der Merwe.

22 December 2010

Z.L Mkhize-Kwitshana

7 February 2011

Prof. Gerhard Walzl

Prof Myra Taylor

Presentations

Part of this work was presented at the following fora:

1. Kwitshana ZL, Taylor M, Dhansay A, Jooste PL, Walzl G. The influence of helminths on immune responses to HIV. 58th American Society of Tropical Medicine and Hygiene Conference, Marriott-Wardman Park, Washington DC. 17-22 November 2009. **Poster presentation.**

2. Mkhize- Kwitshana ZL, Taylor M, Dhansay A, Jooste PL, Walzl G. The immulogical interactions between Helminths and HIV: Implications for HIV pathogenesis. 5th PHASA Conference, International Convention Centre, Durban. 30 November – 2 December 2009**. Oral Presentation**

3. ZL Kwitshana, Fincham JE, Adams VJ, Dhansay MAvan der Merwe L & Walzl G. The influence of helminths on immune responses to HIV. 3rd South African Aids Conference. Durban ICC. 30th May- 2nd June 2007. **Poster presentation.**

4. Z. L. Kwitshana & G. Walzl. The influence of helminths on immune responses to HIV. The Experimental Biology Group. Stellenbosch University. 2005. **Oral presentation**

Acknowledgements

The author is grateful to the following individuals without whom this thesis would not have been produced:

I wish to thank my supervisor, Prof Gerhard Walzl , head-Immunology, Department of Biochemistry, Stellenbosch University for his intellectual input, his expert contribution in immunology, dedicated guidance, encouragement and patience.

I also thank Prof Myra Taylor, co-supervisor and for her kind assistance in thoroughly editing the thesis, her encouragement and patience.

The residents of Khayelitsha for participating willingly in the study.

Dr. Lize van der Merwe for her statistical assistance.

Dr J. Mwamba, the late Dr Ivan Tom, Sister Mandindi for permission to access the Mathew Goniwe Clinic.

Prof P. Bouic for his expert advice on flow cytometry.

Dr Christo Muller for his expert technical assistance with all flow cytometry work.

Prof Jan Bradley (University of Nottingham, Nottingham, U K) for kindly providing *Ascaris lumbricoides* whole worms from Cameroon.

Ms Natalie Nieuwenhuizen (University of Cape Town) for providing *Ascaris lumbricoides* whole worms from South Africa and advice on antigen preparations.

Ms A. Lynch (University of Cape Town) for assisting with the detection of endotoxin levels in crude worm antigen filtrates.

Prof TFHG Jackson, Dr John Fincham, Dr Ali Dhansay, Ms Vera Adams and Ms Celia Anderson who contributed immensely to the implementation of the whole project.

Dr Musa Mabaso for his kind support and assistance.

The South African Medical Research Council for funding the Project.

The National Research Foundation for providing a travel grant.

Lastly I thank my long suffering family: My husband Sindile for his support and my children, Anele, Siyabulela and Nokwanda who had to cope with undue stress without a choice when I had to do the laboratory work in Cape Town. I thank my sisters Qhu, Carol and Thobekile for providing family support during my long absence from home. I also thank my parents for their total sacrifice in order to give us education.

List of Contents

Title i
Dedication ii
Declaration .... **Error! Bookmark not defined.**
Presentations iv
Acknowledgements v
List of Contents vi
List of Tables xi
List of Figures xii
List of Appendices xiii
5List of Abbreviations xiv
Summary xvi
Chapter 1 1
Literature Review: Human immunodeficiency virus (HIV) and helminth co-infection 1
1.1 Preamble 1
1.2 The Th_1 / Th_2 Dichotomy 3
1.3 Overview of the Immune responses to HIV 7
1.3.1 Cellular immune responses to HIV 7
1.3.1.1 $CD4^+$ lymphocytes 8
1.3.1.2 Cytotoxic CD8+ T-lymphocytes 11
1.3.1.3 Natural Killer (NK) lymphocytes 12
1.3.1.4 B lymphocytes 14
1. 3.2 Humoral Responses to HIV 15
1.3.2.1 Antibody responses 15
1.3. 2. 2 Cytokine responses 16
1.4 Helminthiasis and overview of their immune responses 19
1.4.0 Epidemiology of helminths 19
1.4.1 Humoral immune responses to helminth infections 21
1.4.1. 1 Cytokine responses to helminth infections 21
1.4.1.2 IgE antibody 21
1.4.2 Cellular immune responses to helminth infections 22
1.4.2.1 $CD4^+$ T cells 22
1.4.2.2. The role of eosinophils 23
1.4.2.3 The role of B cells 24
1.4.2.4 Natural Killer (NK cells) 25
1.5. Other host factors that affect the host immune response 25
1.6. Proposed mechanisms by which helminths influence HIV pathogenesis 27
1.6.1 Virus entry 28
1.6.2 HIV replication 30
1.6.3 Impaired immune control 32
1.6.4 Modulation of non-parasite responses by helminth infection. 33
1.7 Regulatory T cells in HIV and in helminth infections 35
1.8 Human studies evaluating direct helminth-HIV immunological interactions 35
1.9 Study hypothesis 41
1.10 Study aim 42
1.11. Objectives 42
1.12 Outline of the thesis 42
References 44
Chapter 2 71

Study Design, Specimen Collection and Preliminary Tests....71
2.1 Study's ethical clearance....71
2.2 Study setting and population....72
2.3 Study design....72
2.4 Questionnaire administration and case record file data collection....77
2.5 Specimen collection....77
2.6 Preliminary tests....78
2.6 1. Detection of faecal helminth eggs and/or ova....78
2.6 2. HIV tests and viral loads....78
2.6.3 Full blood counts and IgE tests....78
2.7 Results....79
2.7.1 General characteristics of the participants....79
2.7.2 Prevalence of helminth infections in the Khayelitsha adult study individuals..80
2.7.2 Prevalence of helminth infections in the Khayelitsha adult study individuals..81
2.7.3 Other infectious diseases....81
2.7.4 Full blood counts, serum Total IgE and *Ascaris lumbricoides* specific IgE....82
2.8 Discussion....82
References....86
Chapter 3 88
Ex-vivo peripheral blood lymphocyte phenotyping, eosinophil counts and their relationship to viral load....88
3.1 Introduction....88
3.1.1 Lymphocyte subsets and viral load in HIV infection....88
3.1.1.1 $CD4^{+}$ lymphocytes:....88
3.1.1.2 $CD8^{+}$ cells:....88
3.1.1.3 NK Cells:....89
3.1.1.4 B cells:....89
3.1.1.5 Viral Load:....89
3.1.2 Helminths and peripheral blood cellular distributions:....90
3.1.3 Flow Cytometry....91
3.1.4 Objectives of the chapter....93
3.2 Methods....93
3.2.1 Participants and samples....93
3.2.1.1 Methodology description....94
3.2.1.2 Optimisation....94
3.2.1.3 Procedure for Staining....95
3.2.1.4 Flow cytometry analysis....96
3.2.1.5 Quality control....99
3.2.1.6 Statistical analysis....99
3. 3 Results....99
3.3.1 Summary characteristics of the HIV-1 positive and the HIV-1 negative participants....99
3.3.2 Characterisation of subgroups by helminth infection and HIV status....101
3.3.3 Immunophenotype profiles of subgroups in HIV^{+} and HIV^{-} groups....102
3.3.4 Interactions between variables in subroups....105
3.3.4.1. The HIV-singly-infected ($egg^{-}gE^{lo}$) group does not differ from the HIV and typical helminth co-infected ($egg^{+}IgE^{hi}$) and all dually infected subgroups....105
3.3.4.2. The HIV positive, egg^{+}/IgE^{hi} group shows a tendency to lymphocytopaenia and significant eosinophilia....105

3.3.4.3 Low IgE and presence of stool eggs is associated with higher $CD4^+$ counts, lower viral load and a tendency towards an increase in all lymphocyte populations. 106
3.3.4.4 High IgE was associated with higher viral loads in the HIV positive group 107
High IgE and eosinophilia were associated with lower $CD4^+$ counts in the HIV negative group 108
3.3.6 Correlations between Viral Load, $CD4^+$ and $CD8^+$counts. 114
3.3.6.1 Correlation between $CD4^+$ and viral load 115
3.3.6.2 Correlation between $CD4^+$ and $CD8^+$ absolute counts 115
3.3.6.3 $CD8^+$ vs viral load. 116
3.4 Discussion 117
References. 124
Chapter 4 131
Immunophenotyping of cell surface activation markers on T lymphocyte subsets of HIV positive and negative subgroups with or without helminth co-infection. 131
4.1 Introduction 131
4.2 Methods 133
4.2.1. Selection of Participants 133
4.2.2. Collection and Cryopreservation of PBMCs. 134
4.2.3 Retrieval and Thawing of Frozen PBMCs 135
4.2.4 Enumeration of PBMCs 135
4.2.5 Surface Staining and Flow Cytometric Measurements of Activation Markers 136
4.2.5.1 Methodology and Modification. 136
4.2.5.2 Procedure 136
4.2.5 Flow Cytometry: Acquisition and analysis 137
4.2.6 Gating strategy for lymphocyte subsets 138
4.3 Results 140
4.3.1. Summary activation profiles in HIV^+ and HIV^- groups. 140
4.3.2 Expression of surface activation markers in HIV positive and negative subgroups 144
4.3.3 Activation profile and interactions between subgroups 148
4.3.3.1 The egg^+/IgE^{hi} infection status is associated with significant increased expression of all activation markers in the HIV positive groups 151
4.3.3.2 Stool egg positivity is associated with increased $CD4^+CCR5^+$ and $CD8^+$ $CD38^+$ 152
4.3.4 Correlations between activation markers and surrogate markers of HIV progression. 153
4.3.4.1 Correlation between $CD3^+$ HLA-DR and $CD4^+$ counts 153
4.3.4.2 Correlation between $CD8^+CD38^+$ and $CD4^+$ counts 154
4.3.4.3 Correlation between $CD3^+$ HLA-DR and viral load 154
4.3.4.4 Correlation between $CD8^+CD38^+$ and viral load 155
4.3.5 Comparison of HIV uninfected, helminth negative results with other studies 156
4.4 Discussion 159
4. 5 Conclusions 165
References. 167
Chapter 5 172
Proliferative and cytokine responses in HIV-1 positive and negative subgroups 172
5.1 Introduction 172
5.1.1 Cytokine and proliferative responses in helminth infection 172
5.1.2 HIV infection: cytokine and proliferative responses 174

5.1.3 Hypothetical interactions between HIV and helminth infections 174
5.1.4 Objectives 175
5.2 Methods 175
5.2.1 *In vitro* culture and stimulation of PBMCs 176
5.2.2 Determination of Proliferative Responses to Stimulants in cultured PBMCs: Ki67 intracellular staining 177
5.2.2.1 Anti-Ki67 and anti- CTLA-4 staining 177
5.2.2.2 Flow cytometric detection: Data Acquisition and analysis 178
5.2.3 Detection of cytokine production by PBMCs 181
5.2.3.1 Methodology-Multiplex Human Cytokine Assay 181
5.2.4 Statistical methods 181
5.3 Results 181
5.3.1 Results of Preliminary experiments 181
5.3.2 Proliferation Assay Results: Unstimulated (baseline) and stimulated proliferation responses to mitogen and recall antigens: Ki67 and CTLA-4 expression in HIV^{+} and HIV^{-} groups: 182
5.3.3 Proliferation profile within subgroups of HIV positive and negative subgroups 184
5.3.4. Interactions of proliferation profiles in subgroups of HIV positive and negative subgroups. 185
5.3.4.1 The HIV+, egg^{+}IgE^{hi} group is associated with increased expression of Ki67 proliferation antigen in unstimulated cells. 185
The HIV-, egg^{+}IgE^{hi} and egg^{-}IgE^{lo} subgroups 185
5.3.4.2 The egg^{+}IgE^{hi} group shows a tendency towards impaired proliferative responses to recall and specific antigens but potent mitogen responses. 185
The HIV-, egg^{+}IgE^{hi} group 186
5.3.4.3 CTLA-4 Responses 190
5.3.4.3 The HIV+ egg^{+}IgE^{hi} group is associated with increased CTLA-4 expression in unstimulated cells 190
5. 3.7 Results of Cytokine Production Patterns 191
5. 3.7.1 Cytokine production by unstimulated cells 191
5.3.8 Patterns of cytokine production by unstimulated cells in HIV^{+} subgroups 193
5.3.8.1 Cytokine profiles in the egg^{+}IgE^{hi} and the egg^{-}IgE^{lo} subgroups 193
5.3.8.2 Type 1 cytokines are decreased in the egg^{+}IgE^{hi} group 193
5.3.8.3 The Th$_{2}$ cytokine IL-4 is higher while Th$_{1}$ cytokines (IL-2 and IP-10) and the regulatory cytokine IL-10 are lower in high IgE responder (egg^{+}IgE^{hi} and egg^{-}IgE^{hi}) sugroups. 193
5.3.8.4 The egg^{+}IgE^{lo} individuals have higher IFNγ (type-1), and lower IL-4 (type 2) cytokines. 194
5.3.8.5 Proinflammatory and anti-inflammatory cytokine production by unstimulated cells. 194
5. 3. 9 Cytokine production in antigen-stimulated cells 196
5.3.9.1 Cytokine production in HIV-1 p24-challenged cells 196
5.3.9.2 Comaprison between the dual infected HIV+ egg^{+}IgE^{hi} and HIV singly-infected egg^{-}IgE^{lo}subgroups 196
5.3.9.2 The Th1 cytokines (IL-2 and IFNγ) are lower in HIV-1 p24 stimulated cells of the egg^{+}IgE^{hi} group individuals 196
5.3.9.4 The egg^{+}IgE^{lo} group had higher IFNγ responses to HIV-1 p24 197
5.3.10 Cytokine production by Ascaris worm-challenged cells 199
5. 4 Summary findings 201
5.5 Discussion 202

5.6 Conclusions..210
References. 212
Chapter 6 218
Challenges and limitations of the study. 218
6.0 Introduction 218
6.1 Study Challenges...219
6.1.1 Funding 219
6.1.2 Recruitment of HIV negative individuals 219
6.1.3 Logistics of Laboratory Sample Processing...220
6.1.4 The possible presence of undiagnosed chronic infections.................................220
6.2 Study Limitations 221
6.2.1 Study design 221
6.2.2 Sample size and sampling...221
6.2.3 Lack of adequate numbers of HIV negative controls......................................222
6.2.4 Lack of age-and sex control 222
6.2.5 Types of specimens 222
6.2.6 Cohort heterogeneity...223
References..224
CHAPTER 7 225
Discussion and Conclusions 225
7.0 Overview 225
7.1 The profile of the study participants 227
7.2 Characterisation of T cell subsets, eosinophils and viral loads 228
7.3 Immune activation and HIV- helminth co-infections 229
7.4 Lymphocyte proliferation and cytokine production 232
7.5 In summary: 234
7.6 Concluding remarks 236
Recommendations 237
References 238
Appendices..243
Appendix 1. Box and Whisker plots of lymphocyte subsets for HIV-1 negative subgroups 243
Appendix 2. Expression of CCR5 on $CD4^+$ and CD^+ cells in HIV^- sugroups..........246
Appendix 3 . Expression of CD38 on CD8+ cells in HIV negative subgroups. 247
Appendix 4. Box and Whisker plots for activation markers in HIV^- subgroups......248
Appendix 6 Box and Whisker Plots for proliferation in HIV- subgroups. 252
Appendix 7 Preparation of stimulating antigens and media 254
Appendix 8 Ethical Clearance Document: SA MRC...266
Appendix 89.Ethical Clearance Stellenbosch University......................................267
Appendix 10. Correspondence for Ethics Waiver –UKN Ethics 268

List of Tables

Table 1. The different cytokines, their sources of production and effects on HIV replication in T-cells, PBMC, macrophages and $CD4^+$ cells. 17

Table 2.1 Summary of baseline characteristics of study participants by HIV status who submitted blood and faecal samples 80

Table 3.1 List of monoclonal antibodies and fluorochromes. 94

Table 3.2 Reproducible results obtained from staining in different containers. 95

Table 3.3 Characteristics and immunological indeces for HIV^+ and HIV^- groups 100

Table 3.4 Median values for lymphocytes and viral load in HIV^+ subgroups. 103

Table 3.5 Median values for eosinophils and lymphocyte in HIV^- subgroups. 104

Table 3.6 Significant interactions between subgroups of HIV+ individuals 110

Table 3.7 Proportions of HIV-1+ participants and their HIV disease markers 114

Table 3.8 a Correlation between $CD4^+$absolute counts and viral load. 115

Table 3.8 b Correlation between $CD4^+$ and $CD8^+$absolute counts 115

Table 3.8 c Correlation between $CD8^+$ and viral load. 116

Table 4.1 Combinations of monoclonal antibody-fluorochrome conjugates. 136

Table 4.2 Percentages of activation markers on T lymphocytes of HIV^+ subgroups 142

Table 4.3 Percentage of activation markers on T lymphocytes HIV^- subgroups. 144

Table 4.4 Kruskal wallis Anova an dmultiple comparisons I HIV+subgroups 150

Table 5.1 Culture plate setup for stimulation assays. 177

Table 5.2 Cytokine production by unstimulated cells in HIV+ subgroups 193

Table Table 5.3 Cytokine production by HIV-1 p24 stimulated cells in HIV^+ subgroups 199

Table 5.4 Cytokine production by Ascaris stimulated cells in HIV^+ subgroups 201

List of Figures

Figure 2. 1. Selection strategy for inclusion and exclusion of participants 76
Figure 3.1 Gating strategy for identification of lymphocyte subsets. 98

Figure 3.2. Box and Whisker Plots for lymphocytes in HIV+ groups. 109

Figure 3.3a Grading immunodeficiency by CD4 + count levels. 112

Figure 3.3b Grading of viral load levels in the four subgroups of HIVand helminth infection. 112

Figure 4.1 Gating strategy for lymphocyte activation markers 139

Figure 4.2 Median percentage expression of activation markers in HIV+ and HIV- subgroups 141

Figure 4.3 Histograms for distribution of viral load data in four subgroups of HIV positive subgroups with or without evidence of helminth infection 143.

Figure 4.4 Expression of CD71 in $CD4^+$ and $CD8^+$ lymphocytes. 145

Figure 4.5 Expression of CCR5 by CD4+ and CD8+ cells in HIV^+ groups. 146

Figure 4.6 Expression of CD38 on $CD8^+$ cells in HIV^+ subgroups. 147

Figure 4.7 Box and whisker plots - activation markers in HIV^+ subgroups. 149

Figure 4.8 Scatterplots for activation versus CD4+ counts in HIV+ groups. 154

Figure 5.1 Gating strategy for proliferation markers in lymphocytes. 180

Figure 5.2 Bar graphs of Ki67 in HIV positive and HIV negative subgroups 183

Figure 5.3 Box and whisker plots for proliferation in HIV^+ subgroups 184

Figure 5.4 Increased expression of Ki67 on unstimulated cells. 188

Figure 5.5 Mitogen and antigen responses in a coinfected individual. 189

Figure 5.6 Potent antigen specific responses in a coinfected individual. 190

List of Appendices

Appendix 1 242

Figure 3. 3 Box and Whisker plots for HIV- subgroups

Appendix 2 245

Figure 4. 5b Expresion of CCR5 on HIV- subgroups

Appendix 3 246

Figure 4.6b Expression of CD38 on CD8+ cells in subgroups of HIV- individuals

Appendix 4 247

Figure 4.6 Box and Whisker plots for expression of activation markers in HIV- subgroups

Appendix 5

Table 4.5 b. Correlations between activation markers, CD4+ counts and Viral load**.**

Appendix 6 250

Figure 5.5 Box and Whisker plotsr Ki67 and CTLA-4 expression in HIV- subgroups 251

Appendix 7 253

Preparation of stimulating antigens and media.

Appendix 8 265

Ethical Clearance from the SA Medical Research Council

Appendix 9 266

Ethical Clearance from the Stellenbosch University

Appendix 10 267

Ethics Waiver from the UKZN Biomedical Research Ethics Committee

List of Abbreviations

ADC	Analog to digital converter
ADCC	Antibody-dependent cell mediated cytotoxicity
AIDS	Acquired Immunodeficiency syndrome
ART	Anti-retroviral therapy
BSA	Bovine serum albumin
CCR	CC (Chemokine) Receptor
CD	Cluster Designation
CDC	Centers for Disease Control
CD4bd	CD4 binding domain
CTL	Cytotoxic T Lymphocytes
DMSO	Dimethylsulfoxide
FCS	Fetal calf serum
Fox p3	Forkhead box P3
HIV	Human Immunodeficiency Virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF 4	IFN regulatory factor 4
LFA	Leucocyte Function Antigen
LTNP	Long Term non-progressor/s
LTR	Long Terminal Repeat (sequences)
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
Min	Minutes
nAbs	Neutralizing antibodies
NHANES	National Health and Nutrition Examination Survey
NFAT	Nuclear Factor of Activated T cells
NFKβ	Nuclear Factor kappa beta
NK	Natural Killer (cells)
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered Saline

PHA	Phytohaemaglutinin
PMT	Photomultiplier tube
PPD	Purified protein derivative
RANTES	Regulated on activation, normally T cell expressed and secreted
RPMI	Rosewell Park Memorial Institute (culture medium)
RORγt	Retinoic acid receptor related orphan receptor γt
RT	Reverse-transcriptase
SA	South Africa
SDF (α)	Stromal-derived factor (alpha)
SI	Syncytium-inducing (strains)
STI	Sexually transmitted infections
TB	Tuberculosis
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor alpha

Summary

In South Africa, co-infection with HIV and intestinal parasites is a major challenge in disadvantaged communities who live in densely populated under-serviced urban informal settlements. This pilot cross sectional study evaluates the immunological effects of co-infection with *Ascaris lumbricoides* and *Trichuris trichura* on the immune response to HIV.

The work was a substudy of a prospective double blind, placebo-controlled investigation to test whether regular deworming changes the immune profile of HIV positive individuals with concurrent helminth infection. The substudy has a cross sectional design and presents pilot data that defines immune profiles of HIV-1 positive individuals with and without gastrointestinal helminth (*Ascaris lumbricoides* and *Trichuris trichura*) infection. The hypothesis was that concurrent helminth infection adversely affects immune responses against HIV. It was conducted in an area of high helminth endemnicity and limited infrastructural resources. Individuals with known HIV infection were recruited from an HIV Support Group and HIV negative individuals residing in the same area (for demographic matching) were used for comparison. The substudy was to provide pilot data for future larger scale and possible interventional studies. The current work is limited by the cross sectional design, moderate sample size and practical challenges.

The profile of lymphocyte phenotypes, viral loads, eosinophils, activation markers, expression of the nuclear proliferation antigen-Ki67 and activation regulator antigen CTLA-4 were analysed using flow cytometry in HIV positive and negative subgroups with or without helminth infection. The type-1, type-2 and inflammatory cytokines were analysed using multiplex cytokine array technology. These were correlated with immune responses to HIV. Non parametric statistics were used to describe differences in the variables between the subgroups.

A major finding of the study was the result of the supplementary use of the serological marker, *Ascaris lumbricoides*-specific IgE in addition to the presence (or absence) of helminth eggs in stools to classify intestinal helminth infection status. Two significant outcomes of this measure were the enhancement of diagnosis of current or recent

helminth infection and, more importantly, the distinction of different phenotypes of individuals who displayed different immunological responses to co-infection with HIV and helminths. The different helminth infection phenotypes are defined by stool egg positivity (egg^+) or negativity (egg^-) with either high or low Ascaris-specific IgE (IgE^{hi} or IgE^{lo}) respectively. The four subgroups, egg^+IgE^{hi}, egg^+IgE^{lo}, egg^-IgE^{hi} and egg^-IgE^{lo} showed different interactions with regards to immune response to HIV. It should be noted that no Trichuris specific IgE tests are commercially available but that there is significant antigenic cross-reactivity with Ascaris antigen.

The presence of helminth stool eggs and high Ascaris IgE (egg^+IgE^{hi}) was associated with the following characteristics: reduction in numbers of all lymphocyte populations, frequent eosinophilia, highly activated immune profiles, antigen specific proliferative hyporesponsiveness, impaired type 1 cytokine responses in unstimulated and antigen stimulated cells and increased TNFα levels. In HIV infected individuals, the egg^+IgE^{hi} helminth infection status was associated with lower but not significant $CD4^+$ counts and higher viral loads. A strong negative correlation was observed between viral loads, $CD4^+$ and $CD8^+$ cells in this subgroup.

Subgroups with high IgE (egg^+IgE^{hi} and egg^-IgE^{hi}) had elevated Th_2 markers with lower $CD4^+$ counts and higher viral loads in the HIV^+ group. The inverse correlation between viral load and $CD4^+$ counts found in all the HIV^+ participants was strongest in these two subgroups.

Individuals with parasite eggs in stool and low Ascaris IgE (egg^+/IgE^{lo}) presented a modified Th_2 profile. This subgroup had high absolute numbers of all lymphocyte subsets in both HIV^- and HIV^+ groups with higher $CD4^+$ counts in the HIV^- and lower viral load in the HIV^+ groups as well as higher interferon gamma, lower IL-4 and higher IL-10.

In conclusion, the results suggest that helminth infections may be associated with deleterious effects on the immune responses to HIV in certain groups of susceptible individuals. The underlying reasons for the different stool egg/Ascaris IgE combinations in settings with high exposure to helminthes is currently not clear but genetic predisposition and environmental factors could play a role. Future studies of helminth-HIV co-infection have to ensure adequate definition of helminth infection status by the

use of both stool examination and measurement of helminth-specific IgE as the infection phenotype is associated with differential effects on HIV associated immune responses. This may also apply to co-infection with other pathogens, including tuberculosis. The long-term effect of helminth co-infection in HIV positive people was not assessed in this study but requires further studies.

Chapter 1

Literature Review: Human immunodeficiency virus (HIV) and helminth co-infection

1.1 Preamble

Two thirds of the people infected with the Human Immunodeficiency Virus (HIV) worldwide reside in sub-Saharan Africa while the majority of the two billion individuals infested with helminths are in this same region (UNAIDS/WHO, 2006). In South Africa (SA) the estimates for adult (15-49 years) HIV prevalence remained high at 17,5% of 48,5 million people (the entire population) in 2007 (UNAIDS/WHO, 2008). This makes SA one of the countries with the highest HIV prevalence in the world. Although the national estimates of helminth prevalence are not known, data from surveys in different SA provinces reveal infestation levels ranging between 70.0 -100.0% in school age children and preschoolers (Evans *et al.,* 1987; Fincham *et al.,* 1996; Mosala, 2001; Mabaso *et al*., 2004*;* Saathoff *et al*., 2005; Wolmarans *et al.,* 2005; Fincham and Dhansay, 2006).

Disadvantaged, poor communities who live in densely populated and under serviced urban informal settlements as well as in some rural areas carry most of the disease burden of the two infections (Bradshaw and Steyn, 2002). Co-infection with HIV and intestinal parasites is therefore a major challenge in these settings which comprise a big proportion of the country's population.

Current thinking suggests that chronic infection with helminths exacerbates the HIV epidemic in developing countries by increasing susceptibility to the virus, enhancing its replication, facilitating faster progression to AIDS and making it more severe compared to the disease in the developed world (Bentwich et *al*., 1995; 1997; 1999; Borkow and Bentwich; 2004).

Several aspects of helminthiasis are proposed to contribute to this interaction: Firstly, the large extracellular helminths induce a strong humoral response with a Th_2 bias, while HIV, being an intracellular pathogen initiates a predominantly cell mediated Th_1 response. These two arms of the immune response counter regulate each other such that predominance of one arm precludes effective induction of the other (Janeway *et al*.,

2001). Secondly, helminth infections persist for protracted periods (life expectancy ranges from 1-3 years and 3-5 years for soil transmitted helminths and schistosoma adult worms respectively, with rapid reinfection rates (Maizels *et al.*, 1993), thus inducing a chronic state of background immune activation; HIV replicates better in activated cells (Fauci, 1993; Lawn *et al.,* 2001). Thirdly, helminth infections are documented to result in increased expression of CD4 and CCR5 molecules (Secor *et al.*, 2003), both of which facilitate virus entry into cells. Lastly, helminths induce generalised immune suppression (Borkow and Bentwich, 2004) which increases susceptibility of infested hosts to bacteria and viruses. It is partly on this basis then that infection with helminths is suggested to create a favorable milieu for HIV to replicate unabated (Clerici *et* al., 2001; Lawn *et al.*, 2001; Borkow and Bentwich, 2004). However, some studies elsewhere in the continent have found no evidence of improvement of HIV pathogenesis after deworming of co-infected individuals (Lawn *et al.* 2000; Elliot *et al.*, 2003; Brown *et al.,* 2004; Modjarrad *et al.,* 2005; Hosseinipour *et al.,* 2007) while two have reported a positive impact of worm treatment on HIV viral load and $CD4^+$ counts (Wolday *et al.*, 2002; Kallestrup *et al.,* 2005). A recent systematic review (Walson and John-Stewart, 2007) acknowledges the lack of sufficient evidence to confirm the benefits of helminth eradication in co-infected hosts. However, these authors indicated that data from one randomized clinical trial (RCT) and several observational studies suggest that deworming is of benefit in reducing plasma viral load.

Studies analysing immunological interactions between the two infectious agents have not been documented in South Africa. An indirect association was drawn from deworming of schoolchildren and reduction of eosinophilia (Fincham *et al.*, 2003). The present study is thus based on the hypothesis that co-infection with HIV and intestinal helminths worsens the pathogenesis of HIV. An attempt is made to investigate some aspects of the immune response in the presence and absence of dual infection to ascertain if indeed individuals afflicted with both infections are immunologically-disadvantaged as far as anti-HIV responses are concerned.

1.2 The Th_1 / Th_2 Dichotomy

The Th_1-Th_2 notion defines the ability of the immune response to adapt to antigenic challenge and respond with a specific phenotype for different types of antigens, such as intracellular or extracellular pathogens. This has significant bearing to the current study as it deals with both types of organisms and there is a lot of debate around the role of Th_1 and Th_2 on influencing the pathogenesis of both infections.

In the specific context, $CD4^+$ T lymphocytes differentiate into various functional subsets characterized by the profile of the cytokines they produce. Initially the two subsets (Th_1 or Th_2) were defined and accepted as central to the Th_1/Th_2 dichotomy and constitute the fundamental immune responses. The regulatory T cells, designated T_{reg}, were also described and shown to have immunosuppressive effects (Janeway *et al*., 2001; Fontenot *et al*., 2003). Helminths are documented to strongly induce both the Th_2 and T_{reg} phenotypes (Maizels *et al*., 2004; Diaz and Allen, 2007). Recently, another distinct CD4+ T cell subset has been described, the Th17 cells which produce proinflammatory cytokines and play a role in extracellular bacterial infections and autoimmunity (Kurts, 2007). Diaz and Allen, (2007) have presented a four dimensional map of the immune response profile suggesting that these four cell phenotypes are superimposed and coexist in different disease patterns and counterbalance one another's effects.

Not all the factors that drive the differentiation of the CD4 T cells into the different subtypes are fully understood but several contributors have been indicated such as:

- **The type of MHC: peptide complex**: Peptide antigens that interact strongly with the T cell receptor and reach high densities on the cell surface induce a Th_1 response, while antigens interacting with a weaker affinity and are presented at low concentration induce a Th_2 response (Janeway, *et al*., 2001). Furthermore, since antigen presentation occurs in association with MHC molecules, some MHC: peptide combinations will favour Th_2 while others will have a predilection towards Th_1 responses. Therefore some individuals with certain MHC alleles will be

genetically predisposed to elicit either predominantly Th_1 or Th_2 responses (Janeway *et al.*, 2001).

- **The CD4 molecule:** The absence of the CD4 molecule on T cells was associated with total impairment of Th_2 cell development in CD4 deficient mice and *in vitro* transgenic T cells (Brown *et al.*, 1997). This essential role of the interaction between CD4 and MHC II molecules in differentiation of Th_2 cells was also confirmed by Fowell and colleagues (1997).

- **The co-stimulatory molecules involved:**

For effective induction of an adaptive immune response against the antigen presented to the T cell receptor, ligation of accessory (costimulatory) molecules on the same antigen presenting cell to their respective ligands on the T cell is essential. In the absence of costimulatory engagement, T cells are partially activated and eventually become anergic (Janeway *et al.*, 2001). The influence of costimulatory molecules on the type of immune response that develops has been studied in various models. For example, in mice, the inducible costimulatory molecule (ICOS) was shown to play an important role in the development of Th_2 cells (McAdam *et al.*, 2000).

Ligation of CD40, another costimulatory molecule, reportedly initiates a Th_1 response through IL-12 and subsequent IFNγ productions (McDonald *et al.*, 2002). CD40 blockade in human *in vitro* experiments lead to reduction of the Th_1 cytokines, IFNγ and IL-12 production (Brodskyn *et al.*, 2001), and its critical role on Th_1 cell development had also been shown in mice (Campbell *et al.*,1996; Heinzel *et al.*,1998).

In *in vitro* and *in vivo* mouse and rat models, B7-1 (CD80) was shown to favour Th_1 predominance (Kuchroo *et al.,* 1995; Khoury *et al.*, 1996) while CD28 engagement to B7-2 (CD86) molecule results in Th_2 ascendancy (Ranger *et* al., 1996; Rulifson *et al.*, 1997) and this is said to be protective in the control of Th_1 mediated autoimmunity (Kuchroo *et al.,* 1995; Khoury *et al.*, 1996; Bour-Jordan and Bluestone., 2002).

Engagement of OX 40 (a costimulatory antigen which is upregulated upon activation of T cells) to its ligand is also reported to promote differentiation of Th_2 cells (Rogers and Croft, 2000). These authors also demonstrated that other accessory antigens (the adhesion molecules) also contribute not only to the strength of the activation signal but also to the differentiation of the T cells. Binding of Leucocyte Function Antigen (LFA) -1 to Intercellular Adhesion Molecules 1 and 2 was shown to upregulate Th_1 and suppress Th_2 differentiation.

While the essence of involvement of costimulatory molecules in immune responses lies in ochestrating an effective response that will eliminate the pathogens, it is suggested that some organisms have evolved mechanisms of manipulating expression of particular costimulatory molecules that will favour their survival in the infected host (Brodskyn *et al.*, 2001). The authours demonstrated that *Leishmania major* increased the expression of CD40 and CD80 on macrophages that were co-cultured with lymphocytes. Their interpretation suggested a strategy by these parasites to modulate the immune response to their advantage.

- **The cytokine microenvironment that predominates during antigen presentation to the T cell:** The initial innate response to antigens induces the production of the different cytokines, for example, some intracellular bacteria and viruses will induce dendritic cells and macrophages to secrete IL-12 (Janeway *et al.*, 2001, Rodriguez-Sosa *et al.*, 2002). This cytokine will in turn activate Natural Killer (NK) cells to secrete interferon gamma (IFN-γ). IL-12 and IFN-γ will activate naïve $CD4^+$ T cells to differentiate into the Th_1 phenotype. IFN-γ will inhibit differentiation of Th_2 cells (Janeway *et al.*, 2001). Extracellular pathogens such as helminths will induce expression of CD 1 molecules. These are normally expressed by thymocytes, professional antigen presenting cells and intestinal epithelia. CD 1 molecule expression activates a specialized subset of CD4 T cells known as NK T cells because they express the NK 1.1^+ marker associated with NK cells. The activated NK T cells synthesize and produce IL-4 which induces proliferation of Th_2 cells. IL-4 or IL-10 alone or in combination, will inhibit Th_1 cell proliferation (Janeway *et al.*, 2001). In another scenario, dendritic cells activate $NK1.1^+$T cells via CD1 molecules and lead to production of IL-4 by these. Thus in the presence of IL-12, the cell will be stimulated to develop towards the Th_1 arm while IL-4 will drive

the T cell towards the Th_2 profile (Jordan and Fredrich, 1987; Janeway *et al*., 2001). Th_1 cells produce IFNγ and IL-2, resulting in a predominantly cell-mediated response, while Th_2 cells produce IL-4, IL-5, IL-10 and IL-13 resulting in a mainly humoral response. Intracellular pathogens inside macrophage vesicles will be cleared by mainly Th_1 responses while extracellular pathogens will elicit a predominantly Th_2 response. During the initial interaction with foreign antigens, the innate immune cells will recognise pathogen associated molecular patterns on bacterial surfaces or viral DNA and induce the relevant Th effectors. It is suggested that in the absence of these molecular recognition patterns, as occurs in helminth infections, dendritic cells will secrete transforming growth factor β (TGFβ) which upregulates Forkhead box P3 (Fox P3) and induce differentiation of the regulatory cells (Fontenot *et al*., 2003).

The polarised Th_1/Th_2 arms of immune responses are easily dissected and studied in mouse experiments, however, a mixture of the two responses sometimes occurs *in vivo* in humans, especially with some protein antigens which may produce a mixture of peptides: some that induce Th_1 cells while others induce the Th_2 phenotype (Janeway *et al*., 2001). In that regard, Picker *et al*. (1995) aptly demonstrated that cytokine synthesis networks are more complex in humans than the oversimplified distinction of either Th_1 or Th_2 phenotype.

- **Intracellular Factors**

The different intracellular factors that regulate transcription after the antigen-T cell receptor interaction has initiated a signal, also play a role in determining the fate of differentiating CD4+ T cells. The signal transducer and activator of T cell transcription factor (STAT) 4 and T-bet together with IL-12 promote development of Th_1 clones while GATA-3, STAT 6 with IL-4 promote Th_2 cell development (Usui *et al*., 2006). Th17 cells are induced by different types transcription factors, namely retinoic acid receptor related orphan receptor γt (RORγt), IFN regulatory factor 4 (IRF-4) and STAT3 (Kurts, 2007). Differentiation of T_{regs} is stimulated by Foxp 3, which in turn is upregulated by expression of TGFβ by dendritic cells during the innate immune response (Fontenot *et al*.,2003).

- **Type of antigen presenting cells**

In mice, antigen presented on B cell surfaces optimally induced proliferation of Th_2 cells while the monocyte macrophage lineages induce Th_1 cell clones (Gajewski *et al.*, 1991).

- **Parasite factors**

Finally, there are suggestions that some helminths manipulate the immune response to induce a favourable environment for their continued co-existence in the host. These suggestions support the hypothesis that helminth species have independently evolved several mechanisms to dampen the inflammatory Th_1 response and thus preferentially induce Th_2 biased responses. Maizels *et al.* (2004) in their elaborate review of these mechanisms have demonstrated that several helminth species secrete factors that manipulate dendritic cells to produce less IL-12 and switch to a Th_2 phenotype. Similarly alterations in the macrophage populations are effected by helminths through induction of alternatively activated macrophages, with reduced IL-12 production and increased potential to induce a Th_2 immune response (Rodriguez-Sosa *et al.*, 2002).

1.3 Overview of the Immune responses to HIV

1.3.1 Cellular immune responses to HIV

Infection with HIV induces an adaptive immune response like all viruses. However, the elicited response rarely eliminates the HI virus, but helps to contain it, particularly during the primary and asymptomatic phases of infection. Both $CD4^+$ and $CD8^+$ antigen specific T cells directed against various HIV-1 antigens and peptides have been defined in different experimental settings (Betts *et al.*, 2001; Imani *et al.,* 2002; Boritz *et al.*, 2004). These studies analysed different aspects of the specific responses.The present study is based on total quantitation of T cell subsets in unstimulated whole blood and cytokine production plus lymphoproliferation in response to HIV p24 and helminth proteins (excretory/secretory (E/S) antigen) only and therefore specific responses against different HIV or helminth proteins are not discussed in detail. Frequencies of the respective cells in relation to HIV are discussed briefly.

1.3.1.1 CD4$^+$ lymphocytes

It has been demonstrated that robust HIV-1 specific CD4$^+$ responses, (particularly HIV-1 gag-specific) in HIV infection are critical in generating and maintaining competent cytotoxic T lymphocyte (CTL) functions in viral infections (Matloubian *et al*., 1994; Kalams and Walker, 1998). Paradoxically, the classic immunologic feature of HIV infection is depletion of the very CD4$^+$ lymphocytes from the primary stage through to AIDS disease. Several mechanisms have been proposed to be directly or indirectly responsible for this phenomenon. These include, among others, direct cytopathic effects and virus burden (Connor *et al.,* 1993), functional disorganisation and gradual alteration of regenerative capacity of the immune system (Grossman *et al*., 2006), disruption of CD4$^+$ T cell homeostasis and HIV-induced impaired production of T cells by the thymus (Douek *et al*., 2001; Douek, 2003; Gougeon, 2003) and imbalanced cell proliferation and cell death rate (Ribeiro *et al*., 2002). It is probably a combination of all of the events and many others taking place simultaneously or consecutively that eventually leads to extensive attrition of CD4$^+$ lymphocytes. The common feature of the killing mechanisms is the fact that not only HIV-infected cells are affected, but also uninfected bystander CD4$^+$ cells are depleted. Some of the prominent hypotheses that are suggested to be important contributors to the massive depletion of the CD4$^+$ T cell compartment are briefly discussed below.

- **Immune activation** and associated clonal exhaustion has been strongly implicated to contribute to the decline in the CD4$^+$ T cell population. Although the exact mechanism by which immune activation depletes CD4 T cells during HIV infection remains unclear, this process has been widely cited as a prominent mediator of CD4$^+$ T cell depletion, as activation positively associates with disease progression (Moanna *et al*., 1996; Giorgi *et al*., 1999; Leng *et al.,* 2001; Sousa *et al*., 2002; Hunt, 2007). At basic molecular events, activated CD4$^+$ T cells induce transcription factors that are essential for virus replication. The transcription factor NFKβ, that is present in all activated cells, binds to both host DNA promoter and viral long terminal repeat sequences (LTR) which then initiates viral transcription (Janeway *et al*. 2001). In that way, infected, activated CD4$^+$ T cells can therefore be prone to depletion by the viral infection/replication cycle.

Leng *et al. (*2001) and Sousa *et al.* (2002) showed that $CD4^+$ cell decline is significantly and directly linked to immune activation. This association was shown to be more significant than that of viral load and $CD4^+$ counts. Bahbouhi and co-workers (2004) suggested that preferential targeting of Th_1 $CD4^+$ by replicating HIV could lead to clonal exhaustion of this $CD4^+$ population. The preliminary finding that reduction of activation by administration of immunosuppressant cyclosporin A at initiation of ART improved $CD4^+$ cell restoration compared to ART regimen initiated alone (Rizzardi *et al*., 2002), strongly supports the notion of immune activation-induced $CD4^+$ decline. Cyclosporin A inhibits activation of T cells by countering the NFAT pathway thereby halting transcription of the IL-2, IL-4 and IL-2 receptor genes and thus IL-2 dependent T cell proliferation (Janeway *et al*., 2001). It was also shown that cyclosporin A alters the processing of HIV-Gag proteins (Streblow *et al*., 1998) thus producing non-infective proviruses, therefore the total effects of cyclosporin A incorporation into ART reduces viral replication.

The “runaway hypothesis” suggests the vicious cycle wherein the virus induces immune activation; immune activation enhances viral replication by increasing cell numbers that are susceptible to infection and support replication; as more viral particles are produced they in turn infect and activate other cells (Yates *et al*., 2007). Using a mathematical model to ascertain if this mechanism can solely explain the slow depletion of $CD4^+$ T cells during the chronic phase of infection, the authors concluded that other non-mutually exclusive mechanisms are also at play.

Syncytium formation

In vitro, HIV infected and uninfected $CD4^+$ T cells have been shown to fuse and form syncytia which soon lyse and die (Blanco *et al.,* 2004; Lopez-Balderas *et al.,* 2007). This occurs as infected cells express viral Env proteins on their surface. These proteins interact with the CD4 molecule and the CXCR4 co-receptor on uninfected bystander cells, forming multinucleated giant cells that undergo apoptosis (Castedo *et al.,* 2003). Visual proof of the process *in vivo* has been demonstrated in human lymphoid tissue (Orenstein, 2000) and is associated with disease progression. Syncytium-inducing (SI) viral strains are known to replicate faster than non-SI strains (Callaway *et al*., 1999) thus both the actual viral turnover and the fusion cytopathy

may jointly contribute to the marked decline in CD4^{+} T cells. This may occur faster particularly later in infection as the IL-15 induced transition from non-SI to SI strains takes place during the chronic stage of the disease (Callaway *et al.*, 1999).

- **Increased apoptosis**

  As one of the many strategies of HIV to evade host immune attack, the virus produces pro-apoptotic proteins and downregulates anti-apoptotic molecules (Badely *et al.*, 2000; Zhang *et al.*, 2001;Yang *et al.*, 2003; Gougeon,2003; Minami *et al.*, 2006; Porichis *et al.*, 2007). Both the TNF family and the mitochondrial apoptotic pathways are induced by the HIV proteins in uninfected and infected cells, thus triggering destruction of effective immune control of the virus and contributing to CD4^{+} T cell attrition. The natural host response to increased destruction of T cells is manifested by increased turnover (proliferation and differentiation) (Janeway *et al.*, 2001). However, the production of several proapoptotic molecules by the persistent HIV virus offsets the compensatory T-cell production by destruction of progenitors that express the respective receptors and ligands in the thymus, periphery and bone marrow (Gougeon, 2003).

  In addition to the virus-induced, increased cell death via the extrinsic pathway, the intrinsic pathway which is normally induced via the internal sensors in activated cells, is also triggered because of the persistent activation by the high virus load in the system. Proliferation is also induced on bystander cells with resultant enhanced apoptosis (Tough *et al.*, 1996). It has been repeatedly shown that T cells from HIV infected individuals are more susceptible to increased apoptosis (Gougeon and Montagnier, 1993; Clerici *et al.*, 1996; Gougeon, 2003).

- **Excessive viral protein-induced cytopathic effects**

  Persistent viral replication constantly produces viral proteins that accumulate in the extracellular compartment and bloodstream. This was shown by investigators in early studies of HIV infection: Stevenson and co-workers (1988) demonstrated that doubling of gp120 production enhanced cell death after HIV infection. These proteins, particularly the envelope glycoprotein complex (gp120/gp41) can act as antigens and attach to CD4 molecules on uninfected CD4^{+} T cells. The antigen-coated T cells are in turn attacked and killed by the HIV specific cytotoxic T cells

(CTL) and antibody-dependent cell mediated cytotoxicity (ADCC) (Gougeon, 2003). Cottrezz *et al.* (1997) also demonstrated that gp 120 on antigen-presenting macrophages prime both HIV infected and uninfected T cells to undergo apoptosis.

1.3.1.2 Cytotoxic CD8+ T-lymphocytes

The major histocompatibility (MHC) class-1 restricted cytotoxicT lymphocytes (CTL) have been documented to play a pivotal role in the control and sometimes even prevention of HIV infection. In many studies, HIV-1 specific CTL responses to single, multiple or whole-genome-spanning synthetic peptides and antigens have been well characterised. Subsequently, an abundance of evidence suggests that immune control of HIV infection is dependent on competent CTL or specific $CD8^+$ responses (McMichael *et al.*, 2000; Kiepiela *et al.*, 2004; Addo *et al.*, 2007; Day *et al.*, 2007).

The best direct evidence for the role played by CTL had been repeatedly demonstrated in the animal model (macaques) of the HIV simian counterpart. Depletion of $CD8^+$ cells in these animals resulted in enhanced viral replication and accelerated disease as indicated by increased plasma viraemia (Jin *et al.*, 1999, Metzner *et al.*, 2000). Indirect but strongly suggestive evidence for the critical role of $CD8^+$ lymphocytes in humans was subsequently derived from several case scenarios:

- During the primary phase of HIV infection, a rapid decline of plasma viral load follows the appearance of a CTL response (Borrow *et al.,* 1994; Koup *et al.*, 1994). In *ex-vivo* experiments, Borrow *et al.*(1994) showed that patients who mounted a strong virus specific CTL response effectively controlled viraemia and antigenaemia; while waning HIV-specific CTL immunity precedes progression to full blown AIDS (Carmichael *et al.*, 1993, Koup *et al.,* 1994; Klein *et al.*,1995).
- Long term non progressors (LTNP) have been shown to maintain strong, highly functional HIV-specific $CD8^+$ cellular responses compared to their progressing counterparts (Betts *et al.*, 2006). In another study Valdez *et al.* (2002) showed that LTNP also maintain robust CTL responses to other viruses over and above HIV specific immunity.
- CTL responses are capable of selecting escape mutants which have an increased survival potential (Koup, 1994; Borrow *et al.*, 1997; Kiepiela *et al.*, 2004; Leslie *et*

al., 2004). CTL targeting specific viral proteins such as *Gag* is associated with viral control (Masemola *et al.*, 2004).

- Similarly, HIV seronegative infants born of HIV positive mothers have robust CTL responses (Kuhn *et al.*, 2002).
- Furthermore, a wide variety of populations of exposed but uninfected groups in different countries is detailed by different authors. They include HIV negative sexual partners in discordant couples (Braganza *et al.*, 2001), highly exposed seronegative prostitutes (Rowland-Jones *et al.*, 1998), healthcare workers with occupational exposure to HIV infected blood (Pinto *et al.*, 1995) and injection drug users at high risk (Beretta *et al.*, 1996). All the groups have been shown to elicit CTL responses which are presumably associated with protection against infection. Whether these responses are indicators of exposure, or are adjuncts to other yet undefined
- protective mechanisms or are truly protective on their own, remains to be discerned in rigorous studies. So far the argument for the protective role is supported by the above cases.

Untreated HIV infected individuals eventually progress to AIDS in the presence of high frequencies of $CD8^+$ cells. Recent advances in studies of specific cellular responses have provided explanatory evidence for this phenomenon. Essentially, declining functional attributes of the CTL response such as proliferation (Boritz *et al.*, 2004; Day *et al.*, 2007), lack of fully differentiated memory phenotype (McMichael *et al.*, 2000; Addo *et al.*,2007) and reduced degranulation accompanied by reduced IL-2, IFNγ and perforin production (Betts *et al.,* 2006) have been documented during the chronic phase of infection. These explain the eventual loss of the protective role of the CTL response despite the presence of $CD8^+$ numbers that remain high during disease progression. Therefore, quantitative measurements of $CD8^+$ T cells probably have little prognostic value in determining disease progression.

1.3.1.3 Natural Killer (NK) lymphocytes

NK cells are part of the innate arm of the immune response in all viral infections, including HIV. Activated by IL-12 and inteferons α and β, they exert their effector functions in various manners (Janeway *et al.,* 2001). First they secrete perforin and granzymes and induce programmed cell death through Fas ligand and tumour necrosis

factor receptors to mediate killing of virally infected cells. Activated NK cells also secrete IFN γ which together with IL-12 influence the adaptive immune response towards a Th_1 phenotype for optimum intracellular infection control (Janeway, 2001). NK cells also express chemokine receptors CCR5 and CXCR4 which act as chemoattractants to recruit more effector NK cells to sites of active replication (Kottilil *et al*., 2004). In the presence of antibodies, through their Fcγ receptors, NK cells also mediate antibody-dependent cellular cytotoxic (ADCC) killing of antibody coated cells and immune complexes (Levy, 1993). This subset therefore plays a critical role in early infection to contain viral replication before the adaptive response is invoked and later on act in concert with the secondary specific responses. NK cells have been documented to be susceptible to HIV infection through their capability to express CD4, CCR5 and CXCR4 on their surfaces and contribute to the pool of virus reservoir that is poorly accessible during antiretroviral therapy (Valentin *et al*., 2002; Kottilil *et al*., 2004).

In HIV infection, reports on quantitative assessments of NK cells are inconsistent. In 1994, Brenner and colleagues indicated that no increases or decreases in NK cell numbers had been reported in HIV infection. These authors suggested that those earlier studies which reported increases had used a wrong NK cell marker (CD57). However, in 2000, De Souza and workers found no differences in numbers and function between $CD16^+/CD56^+$NK cells of HIV seropositive and 3 seronegative patients from Thailand, while they reported significant differences between seropositive and seronegative North Americans. Again in 2005 Meier *et al.* reported reductions in total NK cell numbers and function in HIV infected participants. There is therefore a discrepancy regarding the numerical changes in this subset in HIV infection.

There is growing evidence however that consistently confirms phenotypic changes which are accompanied by functional impairment of NK cells in HIV disease. A prominent finding is a phenotypic switch characterised by decreased numbers of the $CD16^+/CD56^+$ pool (reportedly the most potent subset) and increased dysfunctional subpopulations. Functional defects associated with these changes include reduced cytolytic activity, impaired cytokine production, upregulation of expression of inhibitory receptors and CCR5 and diminished perforin secretion (Hu *et al*., 1995; De Souza *et al.*, 2000; Kottilil *et al*., 2004; Mavillo *et al*., 2005; Meier *et al*., 2005). These effects were commonly found

in most viraemic participants (Kottilil *et al.*, 2004; Mavillo *et al.*, 2005), which were reversed to normal by antiretroviral control of virus replication (Kottilil *et al.*, 2004).

1.3.1.4 B lymphocytes

The role of B cells (and inherently the humoral response) with regards to protection against HIV infection and progression to AIDS is still ill defined. Also, to date, it has not been shown that HIV directly infects B cells. Most studies indicate that B cell/HIV interactions occur on the surface through receptors such as CD21 (Moir *et al.*, 2000) and DC-SIGN (Rappocciolo *et al.*, 2006).

Functional and phenotypic disturbances of these cells had been well described from early after the discovery of HIV/AIDS (Lane *et al.*, 1983; Schnittman, *et al.*, 1986). The B cell changes associated with HIV/AIDS include polyclonal activation associated with hypergammaglobulinaemia and impaired specific antibody production (Moir *et al.*, 2001; De Milito *et al.*, 2004), decreased expression of CD21 (Moir *et al.*, 2001), increased susceptibility to apoptosis (Ho *et al.*, 2006), loss of memory cell pool (Viau *et al.*, 2007, Hart *et al.*, 2007), impaired proliferation in response to B cell stimulants (Moir *et al.*, 2001). A common characteristic of B cell dysfunction manifests as autoimmunity in AIDS (Levy, 1993).

The less defined protective role of B cell immunity in HIV is juxtaposed with evidence for its ability to promote infection of $CD4^+$ cells and their contribution to the uninfected reservoir (Moir *et al.*, 2000). Through their CD21 receptor, B cells isolated from tonsils and peripheral blood were found to be equally capable of transferring HIV to activated, HIV negative PBMC's as $CD4^+$ cells (Moir *et al.*, 2000). Similarly, Rappocciolo *et al.* (2006) demonstrated that a proportion of B cells from peripheral blood and tonsils of HIV infected and uninfected patients, expressing DC-SIGN were able to transfect $CD4^+$ cells via this receptor. The authors noted that through their migratory pattern in blood and intimate interaction with T cells, this B cell subset may have a significant role in transferring the virus to $CD4^+$ cells.

Increased turnover of B cells, which is marked in advanced disease, has been demonstrated in the macaque-SIV model (De Boer *et al.*, 2003). Increased expression of the CD5 molecules occurs on B cells as disease progresses. This phenotype has

increased susceptibility to apoptosis (De Milito, 2004) which may explain in part the finding of decreased numbers and percentages of B cells in advanced HIV infection (Grunewald *et al.*, 1999).

1. 3.2 Humoral Responses to HIV

1.3.2.1 Antibody responses

Within 4-8 weeks after infection, neutralising antibody responses against the envelope glycoprotein gp 120, the exposed portion of gp 41, and the core proteins p24/p17 are elicited (Levy,1993). The protective ability of these neutralising antibodies (nAbs) is a contentious issue. Earlier work suggested a protective role, particularly as demonstrated in HIV-1 infected long-term non-progressors. Pilgrim *et al.* (1997) in their comparative study, concluded that neutralising antibodies from LTNPs were more likely to neutralise heterologous and autologous strains than sera obtained from fast progressors. Likewise Zhang and colleagues (1997) reported lower viral load, controlled viral replication and a broader, higher neutralising activity of LTNPs sera compared to sera from fast progressing counterparts. In an animal model, Ciurea *et al.* (2000) showed that neutralising antibodies were capable of suppressing viral replication and clearance in transgenetic mice infected with lymphocytic choriomeningitis virus in the absence of CTL. Further studies attributing antibody responses to long term non-progression were still reported in recent times (Montefiori *et* al., 2001; Burastero *et al.*, 2006).

Around the same early period (1997), other studies associated a negative contribution of humoral response to HIV immunity. ADCC killing mediated by gp120 immune complex-NK cell interaction was strongly correlated with rapid $CD4^+$ decline and high virus load (Skowron *et al.*, 1997). A deleterious effect of antibody response was also shown by Homsy and colleagues (1989) who revealed that AIDS patients elicit antibodies that enhance viral replication through complement receptor interactions rather than neutralising ones.

The generation of escape mutants by neutralising antibodies has been well documented (Tremblay and Wainberg, 1990; Skrabal *et al.*, 2005). These antibodies then become irrelevant to autologous strains within the same host, and within contemporary strains,

thereby casting doubt on the protective role of these antibodies. Recently, Bailey *et al*. (2006) found lower titers of neutralising antibody in sera from LTNPs and HAART exposed patients compared to treatment naïve, viraemic patients. They concluded that neutralising antibodies are not essential for viral suppression in long term non progressors, particularly at low virus levels, suggesting that low copies probable do not reach the stimulation threshold to induce and maintain effective neutralising antibody responses.

In 2004, Chien *et al*. reported that patients with high levels of antibodies directed against the CD4 binding domain (CD4bd) of the HIV-1 gp 120 protein progressed faster than those with lower anti-CD4bd antibodies. The explainatory mechanism is that binding of anti- CD4bd inhibits gp 120 presentation to HIV-1 specific $CD4^+$ cells, with a resultant attenuation of the envelope-specific lymphoproliferation responses.

The above studies indicate that the role of antibody responses in controlling and possibly preventing HIV infection so far remains limited.

1.3. 2. 2 Cytokine responses

The dearth of information on cytokine responses in HIV infection is updating rapidly, thus an exhaustive review would be beyond the scope of this work. A brief overview of key influences of the main ones is therefore outlined below.

Intracellular (viral) infections, including HIV-1, typically induce Th_1 cytokine responses such as IL-12, IL-2, IFNγ and TNFα (Lawn *et al*., 2001). During the innate immune response, virally-infected cells produce interferons (IFN) α and β, which activate and stimulate NK cells which in turn secrete IFNγ, among other functions (Janeway *et al*., 2001). During the primary stage of HIV-1 infection, the expansion of $CD8^+$ cells is accompanied by a rise in IFNγ (McMichael *et al*., 2000; Addo *et al*., 2007) and the HIV-1 gp120 glycoprotein directly induces the proinflamatory cytokine TNF α production by mononuclear cells (Clouse *et al.,* 1991). A host of other cytokine networks are induced during the primary phase of HIV infection, some of which are listed in Table 1 and their effects on HIV replication are summarised therein.

Table 1 The different cytokines, their sources of production and effects on HIV replication in T-cells, PBMC, macrophages and $CD4^+$ cells. Some data was adapted from other studies listed in the original source. Reproduced with permission of JA Levy (Levy, 1993 and the Copyright Clearence Center, Licence Number 2265850206530)

Cytokine	Major source	Effect on HIV replication in[b]:			
		T-cell lines	PBMC	Primary macrophages	$CD4^+$ cells
TNF-α	Macrophages, T cells, B cells, keratinocytes	↑ ↑	↑ ↑	↑ ↑	↓[c] or ↑
TNF-β	T cells, B cells	↑ ↑	↑ ↑	NT[d]	⇞
GM-CSF	Macrophages, T cells	–	↑ ↑	NT	⇞
IL-1	Macrophages, fibroblasts, endothelial cells	↑ or –	NT	NT	–
IL-2	T cells	–	↑	NT	↑
IL-3	T cells	–	NT	↑ ↑	⇞
IL-4	T cells	–	NT	↑ ↑	↑
IL-5	T cells	NT	NT	NT	↑
IL-6	Macrophages, T cells, glia, fibroblasts	–	NT	↑ ↑	⇞
IL-7	Bone marrow stromal cells	NT	NT	NT	⇞
IL-8	T cells, monocytes, keratinocytes, fibroblasts, endothelial cells	NT	NT	NT	–
IL-9	$CD4^+$ T cells	NT	NT	NT	↑
IL-10	T cells, B cells, mast cells	NT	NT	NT	–
IFN-α	B cells	↓ ↓	↓ ↓	↓ ↓	↓ ↓
IFN-β	Fibroblasts, B cells	↓	↓ ↓	NT	↓ ↓
IFN-γ	T cells	↓ or –	↑ or –	↑	↑
TGF-β	Platelets, macrophages, T cells	–	↓	↓ ↓	↓[c] or ↑

[b]Data on acute HIV-1 infection of the cells listed are presented. The number of arrows indicates an increase (↑) or decrease (↓) in virus production. A line through the arrow indicates a slight effect. In limited studies, macrophage-colony-stimulating factor (M-CSF) increased HIV replication in primary macrophages and granulocyte-colony-stimulating factor (G-CSF) enhanced HIV production in $CD4^+$cells. [c]High concentration only. [d] NT not tested.

(Source: Levy JA. 1993. Pathogenesis of Human Immunodeficiency Virus infection. **Microbiological Reviews. 57(1)183-289)**.

It is suggested that as HIV infection progresses, a switch from Th_1 to Th_2 predominance ensues (Clerici and Shearer, 1993; Klein *et al.,* 1997). Using flow cytometry and intracellular cytokine staining, Klein *et al.* (1997) demonstrated this shift at single-cell level. These changes have several negative implications with regards to containing the HIV infection: (i) Th_2 cytokines IL-4 and IL-10 downregulate Th_1 cell proliferation and differentiation (Janeway *et al*., 2001). As alluded to in the preceding section, Th_1 (CTL) responses are critical for virus control (ii) IL-2, (a Th_1 cytokine) is a potent inducer of $CD4^+$ proliferation (Janeway *et al*., 2001; Valdez *et al*., 2003). Its reduction or elimination thus exacerbates attrition of this lymphocyte pool. Increased production of IL-4 has been demonstrated in HIV infected individuals (Waldrop *et al*., 1997; Klein *et al.,*1997) and was also shown to induce phenotypic changes from non-syncytia to syncytia inducing viral strains that are known to be associated with disease progression and poor prognosis (Valentin *et al.,*1998) (iii) Th_1 cytokines (IFNγ and IL-2) were shown to inhibit T cell apoptosis, while IL-4 and IL-10 either enhanced or had no effect on programmed cell death (Clerici *et al*., 1994). In summary, these studies concur with earlier assertions that the Th_1 to Th_2 ascendancy is associated with poor prognosis, or that a balanced Th_1/Th_2 response is associated with better control of viral replication (Imami *et al*., 2002).

It has to be noted, however, that some Th_1 cytokines are documented to favour HIV replication, for instance the proinflammatory cytokine, TNFα (Poli *et al*., 1990; Lawn *et al*., 2001). On the other hand, some scientists express caution about oversimplifying the Th_1/Th_2 dichotomy in humans (Allen and Maizels, 1997). It has been suggested that a mixture of the two phenotypes is likely to occur, when some peptides induce Th_1 and others Th_2 responses during the same infection (Janeway *et al*., 2001).

Others have not found a Th_1 to Th_2 shift in HIV disease progression: In early studies, peripheral blood mononuclear cells (PBMCs) and lymph node mononuclear cells from HIV infected individuals were analysed for constitutive cytokine production. Both IFNγ (Th_1) and IL-10 (Th_2) were expressed at high concentrations at all stages of infection while IL-2 (Th_1) and IL-4 (Th_2) were undetected. No evidence of Th_1-Th_2 switch was demonstrable in this study (Graziosi *et al*., 1994). In that same year, Maggi *et al.* (1994) demonstrated that HIV does not induce a definite Th_1/Th_2 shift, but rather a shift to Th_0 clones producing both types of cytokines. Later, Tanaka and colleagues (1999) reported a decline in IL-2 producing cells without changes in other cytokines and no shift in the Th_1/Th_2 response in HIV infected donors.

In 2000, Sabbaj *et al.* evaluated vaccine induced cytokine production in PBMC of healthy HIV seronegative donors at mRNA and protein levels. All prime-boost candidates secreted both IFNγ and IL-10, > 80% had detectable levels of IL-2, IL-5 and IL-6 while >70% secreted IL-4. Again this study revealed a mixed (Th_1/Th_2) cytokine response in these vaccinated individuals. Recently, Bahbouhi *et al.* (2004) delineated productively infected and bystander (HIV exposed but p24 negative) cells to study the effects of virus replication on cytokine profiles and activation/apoptosis. Their findings suggested that HIV either preferentially replicates in Th_1 rather than Th_0 or Th_2 cells or upregulates Th_1 cytokines. They further suggested that this may result in the depletion of the Th_1 clones. Their findings suggest that later as disease pogresses and when the Th_1 clones are depleted, Th_0 and Th_2 would predominate, a notion that would superficially concur with a Th_1- Th_2 switch.

It is thus clear that the issue of HIV Th_1/Th_2 or Th_0 is more complex and dynamic than previously thought. Therefore as with most aspects of HIV immunology, there is

conflicting evidence regarding the Th_1/Th_2 differentiation during HIV infection and progression to AIDS.

Maek-A-Nantawat (2007) reported that HIV infection is associated with significantly increased IL-17 in CD4 positive and negative cells , and most recently, the TH17 phenotype was shown to be selectively lost in the gut mucosa of HIV infected individuals (Brenchley *et al.*, 2008). This finding partly explains the recurrent gastrointestinal pathology associated with AIDS disease because the Th17 cells play a critical role in immune responses to extracellular bacteria. More research on the role of these cells in the pathogenesis of HIV was still ongoing when these findings were published.

1.4 Helminthiasis and overview of their immune responses

1.4.0 Epidemiology of helminths

- **Distribution and prevalence**

Approximately two billion people worldwide are infested by soil transmitted helminths that include the geohelminths, schistosomes and filarial worms, the vast majority of whom are in developing countries (World Health Assembly, 2001) and individuals who are affected, frequently harbour more than one species of worm (Bundy *et al.,* 2000). School-age children and preschoolers are documented to carry the heaviest burden of helminths, hence morbidity and mortality of sTH infections have always been associated with children (Drake *et al.* 2002). The age dependent distribution of helminths has been cited by some to be a result of the development of immunity over time from repetitive exposures (Maizels *et al.*, 1993).

The majority of these infections vary from light to moderate, while 80% of the heavy infections are carried by 20% of the infected population (Maizels *et al.*, 1993). Among others, genetic predisposition to high worm burdens has been reported to be responsible for the overdispersed distribution of these infections (Quintell, 2003).
It is noted that some local studies-Western Cape (Adams *et al.*, 2006) KwaZulu Natal (Kwitshana *et al.*, 2008) have revealed high prevalence of helminth infections in adults,

suggesting that under conditions of poverty, overcrowding and poor sanitation, these infections are just as high in adults as in children.

- **Life expectancy and transmission**

These vary from species to species, for example adult schistosome worms can live for up to five years in the host in the absence of chemotherapy. *Onchocecaria volvulus* has a life expectancy of up to 8-10 years while *Ascaris lumbricoides* is one year. Those with a short life cycle have a faster re-infection rate after chemotherapy and are highly fecund, for example an adult Ascaris female can produce 200 000 eggs per day (Maizels *et al*., 1993).
Geohelminths cycle via pollution of the soil (earth) by human faeces. In the case of schistosomes, water is polluted by urine and/or faeces. These epidemiological cycles have been exacerbated because large numbers of people are living in densely populated slums (informal settlements) without adequate sanitation or clean water, owing to rapid urbanisation and migration (Bundy *et al.,* 2000). In developed countries, the general availability of clean water and effective sanitation has eliminated most of these parasites. Climate, soil and other geographical parameters determine the optimum transmission of different parasites (Mabaso *et al*; 2004). In addition, other factors such as personal hygiene, occupational behaviour and level of exposure also determine infection and transmission.

Lastly, the key features of gastrointestinal nematode infections that are intriguing were aptly described by Maizels *et* al., (1993) and include:

- The persistent nature of these infections despite evidence of host immune responses which implies that the parasites have evolved effective immune evasion mechanisms.
- The size and complex life cycles of these parasites means that the host is challenged by many and varied parasite antigens for protracted periods of time.
- The non-specific immunosuppression which is a universal feature of helminth infections. This may largely explain the increased susceptibility to bacterial and viral infections in parasite-infested individuals.

1.4.1 Humoral immune responses to helminth infections

1.4.1. 1 Cytokine responses to helminth infections

Classically, the immune response to intestinal nematodes (helminths) displays Th_2 type cytokine predominance, characterized by elevated total and parasite-specific immunoglobulin (Ig) E and IgG, eosinophilia and increased mast cells (Rothenberg, 1998; Faulkner *et al.,*2002; Geiger *et al.,*2002). This predominance of Th_2 cytokines has been suggested by some to be closely associated with a protective immune response in both animals (Else and Finkelman, 1998, Roitt *et al*., 1990) and humans (Maizels *et al.,* 1993; Turner *et al*., 2003; Jackson *et al*., 2004; Lertanekawattana *et al*., 2005).

Typical cytokines involved in parasite immunity include IL-4, IL-5, IL-9, IL-10 and IL-13 (Gopinath *et al*., 2000;Turner *et al*., 2003; Borkow and Bentwich 2004; Jackson *et al*., 2004; Maizels *et al*., 2004 ; Lertanekawattana *et al*., 2005). It is not clear what the key role of this Th_2 dominance is. One school of thought holds that it is to protect the host from re-infection (Else and Finkelman, 1998; Turner *et al*., 2003; Jackson *et al*., 2004; Lertanekawattana *et al*., 2005), while others suggest that it is a parasite-induced mechanism of escaping inflammatory Th_1 attack or a combination of host-parasite induced survival strategies (Yazdanbakhsh *et al***.,** 2001;Maizels and Yazdanbakhsh 2003).

1.4.1.2 IgE antibody

IgE is the classic humoral component of host protective immune response against the helminthic parasites (Roitt *et al*., 1990; Rihet *et al*., 1991; Dunne *et al*., 1992). Proteases released by helminth parasites for tissue invasion promote Th_2 responses and hence IgE production (Janeway *et al.* 2001), therefore levels of this antibody are markedly increased in parasitic infestation. The mechanism of parasite killing involves eosinophils and mast cells. Parasite specific IgE coats parasites and renders them susceptible to killing by eosinophils as described in 1.4.2.2 below. Mast cells degranulate after their receptors are cross-linked by IgE. Release of substances such as eosinophil chemotactic factor (ECF), prostaglandins and mucus-inducing agents ensues. IgE is widely documented to be associated with protection against helminth reinfection (Rihet *et al*., 1991; Dunne *et al*., 1992; Faulkner *et al.,* 2002; Geiger *et al.,* 2002; Brake, 2003;

Maizels and Yazdanbakhsk, 2003; Turner *et al.*, 2003; Jackson *et al.*, 2004; Lertanekawattana *et al.*, 2005)

Two mechanisms involved in IgE production include the induction of Th2 responses and initiation of isotype switch to IgE. Both are derived from activated $CD4^+$ Th_2 cells. The DNA rearrangements that occur during isotype switching from $\mu^+\delta^+$ to ε^+ are influenced by the cytokines secreted by $CD4^+$ Th_2 cells, particularly IL-4 (Janeway *et al.* 2001). Production of IgE is thus dependent on IL-4. Mutations in the α chain of IL-4 as well as gain in function, are associated with excessive IgE production.

Other antibodies play a role in helminth parasite immunology and these include the IgG subclasses, IgM and IgA (Janeway *et al.*, 2001).

1.4.2 Cellular immune responses to helminth infections

In principle, several cells interact in orchestrating the overall response and their roles during helminth infections are briefly described below.

1.4.2.1 $CD4^+$ T cells

$CD4^+$ T cells play a critical role in the control of intestinal helminth infection. Upon stimulation by helminth antigens, T lymphocytes are generated early in infection and elicit a secretory response (Osborne and Devaney, 1998; Betts *et al.*, 2000; Brake, 2003). The eosinophil stimulation promoter (ESP) is secreted by the activated T cells, which in turn stimulate eosinophil proliferation. Similarly, the lymphocyte chemoattractant factor (IL-16), which is produced by activated $CD4^+$ lymphocytes, binds eosinophils via CD4 and induces their migration (Rand *et al.*, 1991; Ferland *et al.*, 2004). Furthermore, the activated T cells act on mucus-secreting goblet cells to facilitate helminth expulsion from the gut (Roitt *et al.*, 1990, Brake, 2003).

Several animal models of helminth infections have revealed the pivotal role of $CD4^+$ T cells during intestinal helminth infections. In nude athymic mice, no IgM, IgG and IgE antibodies were detected and helminth expulsion was delayed in these T cell-deficient mice (Vos *et al.*, 1983). Similarly, it was shown that recruitment and development of

other cells such as intestinal mast cells and goblet cells are dependent on T cells (Vos *et al.*, 1983; McDermott *et al.*, 2001). Furthermore, $CD4^+$ cells alone were shown to effect helminth expulsion and confer resistance to *Trichuris muris* in complete absence of antibody (Else and Grencis, 1996). Additionally, deletion of $CD4^+$ T cells resulted in abrogation of protective immunity in BALB/c mice infected with the helminth *Heligmosomoides polygyrus* (Urban *et al.*, 1991). These authors concluded that $CD4^+$T cells regulate host protective immunity, helminth fecundity and IgE levels. Prowse *et al.* (1978) also illustrated that nude (hypothymic) mice failed to clear *Nematospiroides dubious* helminths and injection of T cells into the nude mice positively correlated with increased antibody levels. In mice, schistosomiasis antigens invoked a $CD4^+$ T cell-driven response towards Th_1 with more severe pathology or towards a Th_2 with milder form of pathology (Stadecker *et al.*, 2004). These studies show that a full complement of T cells is essential for development of resistance to the infective stages of the helminths.

Although the plethora of information on the important role played by T cells in intestinal helminth infections has been derived from laboratory animal models (Prowse *et al.*, 1978; Vos *et al.*, 1983; Urban *et al.*, 1991; Else and Grencis, 1996; Else and Finkelman, 1998), human studies have also confirmed these findings. In 2003, Turner and co-workers undertook a cross-sectional community study in which they showed that Th_2 cytokines were associated with reduced helminth burdens with increasing age. Furthermore, in their review of the role of IL-13 (a Th2 cytokine) Grencis and Bancroft (2004) re-affirmed that $CD4^+$ Th_2 cells play a central role in generating protection in both laboratory systems and humans in intestinal nematode infections. Evidence of decreased $CD4^+$ T cells was reported in humans chronically infested with intestinal helminth parasites (Kalinkovinch *et al.*, 1998).

1.4.2.2. The role of eosinophils

Eosinophils comprise 2-5% of the total leucocyte pool in healthy, non-atopic individuals. These cells are key effector cells which play a specialized role in immunity to helminths (Janeway *et al.*, 2001). Although they are capable of phagocytosing and killing ingested microorganisms, this does not appear to be their primary function (Roitt *et al.*, 1990). Their ability to degranulate and release their cytotoxic granule contents (mainly the Major

Basic Protein-MBP) and kill their targets, is best suited for the large targets such as helminths which cannot be phagocytosed (Roitt *et al.*, 1990).

Eosinophil differentiation from the pluripotent stem cell is induced by IL-5, and their attraction to tissues is controlled specifically by eotaxin-1 and eotaxin-2 (Rothenberg,1998) and IL-16 (Ferland *et al.*,2004) although other non-specific cytokines such as the platelet activation factor, leukotriene B-4 and granulocyte-monocyte colony stimulating factor are also chemotactic for eosinophils (Roitt *et al.*, 1990). Parasite killing by eosinophils is effected by antibody dependent cytotoxicity through release of toxic granule contents (Brake, 2003). Eosinophils can adhere to schistosome helminths, degranulate and release cytotoxic granules thereby killing the schistosomules (Roitt *et al.*, 1990).

Eosinophils interact with a variety of cells and IgE to mount the effective responses to helminths. Macrophages, being the first cell line of defense, interact with parasite antigens and present them to T cells (Rodriguez-Sosa *et al.*, 2002). Parasite-activated T cells produce the stimulatory cytokines such as IL-5, which induces differentiation of eosinophils from the pluripotent stem cells (Maizels and Yazdanbakhsk, 2003). The eosinophil stimulation promoter (ESP) which induces eosinophil proliferation is also produced by parasite-antigen specific T cells. Mast cells that are sensitized by anti-parasite-IgE degranulate and secrete eosinophil-chemotactic factors (Brake, 2003; Maizels and Yazdanbakhsk, 2003).

1.4.2.3 The role of B cells

Many parasite antigens act as B cell mitogens, resulting in polyclonal B cell stimulation and proliferation. Subsequently, in addition to high levels of parasite-specific IgE, non-specific hypergammaglobulinaemia occurs in parasite infections (Hagel *et al.*, 2003) Characteristically, total IgE (as well as IgM and IgG) rise above normal levels. Such polyclonal stimulation can result in diminished levels of specific IgE antibody. This is viewed as a means of evading the host's response by the parasites (Lynch *et al.*, 1998).
The continuous antigen exposure and polyclonal stimulation of B cells is believed to lead to impairment of B cell function, progressive depletion of antigen-reactive B lymphocytes

(clonal exhaustion) and thus immunosuppression (Hagel *et al.*, 2003, Maizels and Yazdanbakhsk, 2003).

1.4.2.4 Natural Killer (NK cells)

NK cells form part of the large granular lymphocytes and are phenotyped by expression of the CD16/CD56 molecules on their surfaces. Their role in helminth infections is not well defined. However, a few studies have reported associations between NK cells and helminthiasis. Enhanced NK cell activity was demonstrated in murine trichinellosis (Niederkorn *et al.*, 1988), while a marked concentration of these cells in pleural cavities of mice with filariasis was observed (Korten *et al.*, 2002). The same study suggested a potential cytotoxic function of the NK cells due to their direct adherence to adult helminths. In humans, a case of NK cell lymphocytosis associated with chronic strongyloides infection was reported by Myers and co-workers (2000).

In human schistosomiasis, different correlations between NK cell activity and infection were reported. While Feldmeier *et al.* (1985) reported decreased NK cell function with increasing helminth burden among infected children in Sudan, Barsoum and colleagues (1984), however did not find changes in circulating NK cell activity in two parallel studies in Egypt and Brazil. On the other hand, upon reviewing relevant literature Wynn and Cheever (1995) concluded that NK cells play a critical role in controlling granuloma formation by schistosome eggs. The suggested mechanism is through early production of interferon gamma by these cells with a resultant down regulation of Th_2 cytokines.

These inconsistent reports warrant further investigation of the role played by this population of cells in helminth infections.

1.5. Other host factors that affect the host immune response

- **Nutritional status**

  Malnutrition is recognised as the most common cause of immunodeficiency (Bachou *et al.*,2006)). The most common forms being protein-energy and micronutrient deficiencies are associated with impairement of all the critical

functions of the immune system such as antibody and cytokine production, cell mediated responses, phagocytosis, complement system, and secretory IgA (Cunningham-Rundles *et al.*,2005).

- **Immunosenescence**

  The natural decline of immune competence brought about by aging is associated with prolonged exposure to various antigenic challenge, DNA oxidative stress, thymic atrophy, impaired haematopoiesis, alterations in T and B quantity and function, changes in both innate and acquired immunity which all result in the impaired capacity to respond to infection by the elderly (Carusa *et al.*, 2009).

- **Microbial translocation**

  The migration of the gut flora to other organs has been implicated as a cause of systemic immune activation. Disruption of the intestinal mucosal barrier as occurs in haemorragic shock, thermal injury, malnutrition, antibiotic administration, cytotoxic drugs and total parenteral nutrional supplementation facilitate the translocation of the gut microorganisms to other sterile sites and cause generalised immune activation and inflammatory processes. (Ancuta *et al.*, 2008). The mechanism was earlier confirmed by Brenchely and colleagues (2006) in both animal model of Simian immunodeficiency infection of macaques and chronically HIVinfected humans.The overall outcome is compromised responses to infections and septicaemia that can sometimes be fatal.

- **Genetic predisposition**

  The association of some diseases with possession of certain HLA antigens (such as ankylosing spondylitis and HLA-B7) is an example of the immunologic genetic predisposition phenomenon that has been described to explain differences in susceptibility to various infections in different individuals (Roitt *et al.,* 1990).

- **Other pathophysiologic factors** such as chronic stress, which persistently stimulates the pituitary-hypothalamic-adrenal-axis and increased glucocorticoid circulation (Reiche *et al.*, 2004), prolonged alcohol abuse and pregnancy also result in an impaired immune system of the host.

1.6. Proposed mechanisms by which helminths influence HIV pathogenesis

The central events in helminth infections that are proposed to be co-factors in HIV pathogenesis and transmission are:

- Persistent exposure of the host immune system to parasite antigens resulting in chronic activation
- Generation of a strong humoral (Th2 biased) response
- Induction of generalised and specific immunosuppression and increased apoptosis

Such a milieu of background immune activation and dysregulation is suggested to contribute in various ways to HIV entry, replication and dissemination, and some of the underlying mechanisms as documented are discussed briefly.

Chronic immune activation resulting from prolonged exposure to helminths is widely described by the Israeli workers who extensively studied the immune profile of the thousands of Ethiopian immigrants, the majority of whom were infested by helminths upon their arrival in Israel. Their work, which originated in the ninenties (Bentwich *et al.*, 1995, 1996, 1997, 1999, 2000; Shapira-Nahor *et al.*, 1998; Borkow *et al.*, 2000; Kalinkovinch *et al.*, 2001; Leng, *et al.*, 2001) and a review by Borkow and Bentwich, 2004 propose evidence for an adverse impact of helminthiasis on HIV pathogenesis. In these studies, evidence for helminth infection-induced activation was shown by increased expression of activation markers HLA-DR on CD3, $CD4^+$ and $CD8^+$, and increased levels of CD38 on $CD8^+$ cells and CTLA-4 on T cells as well as neopterin and β_2 microglobin. A strong association between chronic activation by helminths and dramatic spread of HIV has also been suggested in other settings as well (Burkina Faso, Ghana, Guyana, Uganda, Nigeria, Sierra Leone, Kenya, Italy and North American residents (Rizzardini *et al.,* 1998; Clerici *et al.*, 2000, 2001; Gopinath *et al.*, 2000; Secor *et al.*, 2003).

Simultaneous initiation of antiretroviral therapy (ART) and administration of cyclosporin A improved CD4 counts restitution in HIV infected individuals compared to their ART-only counterparts (Rizzardi *et al.*, 2002). This finding strongly supports the negative

relationship between immune activation and HIV pathogenesis, and implications thereof for helminthiasis-induced HIV aggravation.

The activated state in immune cells is postulated to enhance viral replication, transmission and disease progression by various mechanisms. Examples include altered cytokine environment, increased HIV receptors (chemokine receptors and CD4 molecules), increased cell anergy and increased apoptosis resulting in decreased antiviral activity (impaired CTL and chemokines). Indeed immune activation was demonstrated to be a determinant of HIV progression (Mahalingam *et al*., 1993; Giorgi *et al*., 1999) while also, immune activation was shown to correlate better with disease progression (as measured by CD4 decline), than viral load (Leng *et al*., 2001). Kassu *et al*., 2003 showed that treatment of intestinal parasites in Ethiopians resulted in reduction of the activation, thus providing strong suggestive evidence for the link between helminth infection and predisposition to HIV, and severe pathogenesis after such infection.

1.6.1 Virus entry

HIV uses the CD4 molecule and the β chemokine receptor CCR5 for entry into cells to initiate infection. Upon receiving stimulatory signals, CD4 molecules are increased on the cell surface and remain high on activated cells (Fauci, 1993, Janeway *et al*., 2001).

Increased expression of CCR5 associated with immune activation in areas of high parasitic endemicity has been reported. Clerici *et al*. (2000) compared expression of this receptor on PBMC's of Africans and Italians living in rural Uganda to levels of expression on similar population groups living in Italy. Immunophenotyping and mRNA PCR of CCR5 showed elevated CCR5 levels in Africans and Italians living in Africa but not in Africans living in Italy. The authors concluded that the increased CCR5 expression was not genetic but environmentally-driven, and is a determinant of the prevalent R5 virus strains in sub-Saharan Africa. Likewise, Kalinkovinch and colleagues (1999, 2001) ascribed increased CCR5 expression to activation associated with chronic helminth infection as well as a possibility of a genetic trait among immigrant Ethiopians moving to Israel from an area of high parasite prevalence. Secor *et al*. (2003) also reported increased expression of both CCR5 and CXCR4 on $CD4^{+}T$ and mononuclear cells

among schistosomiasis-infected Kenyan car washers compared to those who had been treated.

Under conditions of abnormal immune activation, CCR5 may also be increased on uninfected cells, thereby increasing their susceptibility to HIV infection (Wahl *et al.*, 1999). Sozzani and colleagues (1998) also confirmed by an *in vitro* study that IL-10 increases the expression of CCR5 on monocytes and also enhanced the productive infection of these cells by the macrophage-tropic HIV Bal strain. Furthermore, an animal model of helminth infection showed that these parasites induce a subset of alternatively-activated macrophages which are characterised by (*inter-alia*), increased expression of CCR5 during the chronic phase of infection (Rodriguez-Sosa *et al.*, 2002).

Eosinophils are the most prominent cellular component of parasite immunity, hence helminth infections are the main cause of eosinophilia in developing countries (Rothenberg, 1998). Earlier studies had shown that eosinophils express CD4, bind the HIV gp120 protein and are indeed infectable by HIV (Lucey *et al.*, 1989; Freedman *et al.*, 1991). This is in keeping with the finding that HIV-1 infected eosinophils transiently expressed the viral core protein p24 and the subsequent conclusion that these cells may act as host cells for HIV-1 (Conway *et al.*, 1992). IL-16, a $CD4^+$ cell chemoattractant, was shown to increase surface expression of the CD4 molecules on eosinophils while also promoting their migration (Ferland *et al.*, 2004). It is therefore possible that during chronic helminth infection, recruitment of activated eosinophils to the genital and rectal tracts may provide an additional entry portal for HIV during hetero-and homosexual contact respectively.

Thus it is clear that helminth-induced increased expression of the CCR5 co-receptor on lymphocytes, macrophages, and eosinophils, (all of which are known to be host cells for HIV) combined with increased expression of CD4 molecules on lymphocytes, monocytes and eosinophils may enhance virus entry and transmission.

Furthermore, immune responses to *Schistosoma haematobium* are suggested to enhance HIV transmission and pathogenesis. The pathological sequelae of schistosomiasis by egg sequestration in the genital tracts are hypothesised to increase virus shedding to semen through increased inflammation (Leutscher *et al.*, 2000) and in

female genital schistosomiasis syndrome (Poggensee and Feldmeir, 2001; Mosunjac *et al.*, 2003).

Based on these reported studies, and the findings that (i) CCR5 is preferentially expressed more on $CD45RO^+$ (memory) than CD45RA (naïve) T lymphocytes (Bleul *et al.,* 1997; Kalinkovinch *et al.*, 2001), (ii) HIV replicates better on memory than on naïve lymphocytes (Fauci, 1993; Spina *et al.*, 1997), (iii) marked increase in the proportion of $CD4^+$ $CD45RO^+$ occur during chronic helminth infections (Kalinkovinch *et* al., 1998) support the proposition that chronic immune activation in persons living in regions of high helminth prevalence may be responsible for increased CCR5 expression that subsequently enhances virus spread. However, findings by Patterson and colleagues (1998, 1999), which correlate immune activation with downregulation of CCR5 expression, (downregulated by IL-10) is by our inference contradictory. Whether the fact that these authors were using PBMC's isolated from individuals residing in areas of low / no helminth infections is relevant in the context of helminth-induced upregulation of CCR5 is debatable. Sozzani *et al.* (1998) acknowledged the divergent role of IL-10 in enhancing or downregulating expression of chemokine receptors under different conditions.

1.6.2 HIV replication

HIV relies on the host cell transcription processes to complete its life cycle and produce new virus particles (Janeway *et al.*, 2001*).* The cellular nuclear factor (NF–Kβ) is present in all activated cells and induces transcription of HIV upon binding to its promoter long terminal repeat (LTR) region (Janeway *et al.*, 2001). Also, the nuclear factor of activated T cells (NF-AT) stabilizes the postfusion HIV complex and facilitates reverse transcription of the virus (Lawn *et al.*, 2001). Proinflammatory cytokine signaling pathways, particularly TNFα, IL-1 and IL-2 induce these transcriptional factors (Janeway *et al.*, 2001). IL-6 also synergises with TNFα in monocytes to enhance HIV replication (Gopinath *et al.,* 2000; Lawn *et al.*, 2001). Increased levels of proinflammatory cytokines such as TNFα and IL -2 are well documented in chronic helminthiasis (Bentwich *et al.*, 1995, Henri *et al.*, 2002).

The influence of Th_2 cytokines on HIV replication is supported by evidence demonstrating that HIV replicates better in Th_2/Th_0 than Th_1 cells (Maggi *et al.*, 1994). In addition, potentiation of Th_2 cytokines during helminth infection has negative consequences with regards to enhancing viral replication: Th_1/Th_2 reciprocal cross-regulation would promote viral replication as predominance of Th_2 cytokines (IL-10 and IL-4) which, alone or in combination inhibit Th_1 cell proliferation, thereby impairing potent innate and CTL responses that are well documented to be critical in control of HIV replication. Furthermore, increased levels of regulatory T cells (T_{regs}) are reported in helminth infections (Borkow and Bentwich, 2004; Mckee and Pearce, 2004). These cells secrete high levels of IL-10 and transforming growth factor β (TGF β).The latter cytokine was shown by Chen *et al*. (2005) to enhance virus entry by increasing expression of CXCR4 in macrophages. Likewise, Sozzani and colleagues (1998) showed that IL-10 increases expression of CCR5 and infection by HIV in human monocytes.

However, it is noted that the role of Th_2 cytokines (IL-4 and IL-10) on HIV replication is contentious. While by virtue of decreasing CCR5 expression on $CD4^+$ cells (Patterson *et al.*, 1999), IL-10 would thus impact negatively on virus entry and replication. On the other hand Weissman and co-workers (1995) demonstrated that IL-10, in synergy with other cytokines enhances HIV production on PBMC's. Furthermore, IL-10 also inhibits pro-inflammatory processes initiated via the NFk-β transcription (Schottelius *et al.*, 1999), and could thus inhibit viral replication. IL-4 was also demonstrated to increase CXCR4 and decreasing CCR5 expression on human CD4 T cells and induce a switch from non-sincytium (NSI), to SI inducing (rapidly-replicating) strains (Valentin *et al.*, 1998) and this was interpreted to exacerbate HIV progression.

For the reason that HIV uses the host's transcription machinery to complete its cyle, it is thus clear why the virus replicates more effeciently in activated than in naïve cells (Fauci, 1993). The finding that CCR5 expression is increased in activated cells (Kalinkovinch *et al*. 2001), and that helminth infections induce both cellular activation (Kalinkovinch *et al*. 1998; Borkow and Bentwich, 2004) and upregulation of CCR5 (Bleul *et al.,* 1997; Kalinkovinch *et al.*, 2001) is in support of the augmentive interaction between helminth infection and HIV transmission.

1.6.3 Impaired immune control

Chronic helminthiasis is associated with generalised and antigen specific attenuation of effective immune responses (Borkow *et al*, 2000; Borkow and Bentwich, 2004). Several cellular and molecular mechanisms have been described.

Effective T cell activation requires appropriate antigen presentation to the T cell receptor together with ligation of the costimulatory molecule, CD28 on T cells to B7-1 (CD80) or B7-2 (CD86) on the same antigen-presenting cell (Janeway *et al*., 2001). CD28 enhances IL-2 mRNA stabilisation and IL-2 R expression by T cells. IL-2 then acts in an autocrine mode to induce T cell proliferation during activation. T cell receptor engagement in the absence of CD28 results in impaired T cell proliferation, activation and permanent anergy (Janeway *et al*., 2001). Major decrease in the number of $CD8^+CD28^+$ T cells during helminth infection has been documented, as well as low CD80 expression on antigen presenting cells (Kalinkovinch *et al*., 1998; Borkow and Bentwich, 2004).

Another downmodulatory feature associated with helminthiasis is upregulation of the counter-stimulatory molecule, cytotoxic T-lymphocyte associated antigen-4 (CTLA-4). CTLA-4 increases the threshold at which T cells are stimulated by reducing IL-2 production (Janeway *et al*., 2001). This is a natural process to dampen immune activation to prevent pathological consequences of uncontrolled activation. CTLA-4 binds to B7 ligands with higher affinity compared to CD28 (Janeway *et al*., 2001). Levels of CTLA-4 are documented to be elevated during helminth infections (Borkow *et al*., 2000; Steel and Nutman, 2003).

Binding of CTLA-4 also induces production of transforming growth factor β (TGF β) (Borkow and Bentwich, 2004). This downregulatory cytokine, together with IL-10 is also produced by T suppressor ($CD25^+$ $CD4^+$) T-regulatory cells, which are known to be also increased during helminthiasis (Mckee and Pearce, 2004; Borkow and Bentwich 2004). Chen *et al*., (2005) demonstrated how TGF β enhances HIV replication by increasing CXCR4 expression, increasing macrophage responsiveness to stromal-derived factor-α (SDF-α) and enhancing virus entry. Increased C1b-1, which is an intracellular negative regulator of activation, also occurs in helminth infection (Borkow and Bentwich, 2004).

Chronic T cell hyperactivation also results in functional anergy, thus preventing the host to respond to cognate antigens (Giorgi *et al*., 1999) to the advantage of HIV replication.

In addition, completion of T cell activation also involves phosphorylation and dephosphorylation of kinases to facilitate translocation of transcription factors to the nucleus for transcription of relevant genes (Janeway *et al*., 2001). Helminth infection was associated with a markedly aberrant process of phosphorylation/dephosphorylation of tyrosine kinases, defective degradation of IκBα, and total impaired signal transduction (Borkow *et al*., 2000). Partial activation of T cells induces anergy (Janeway, 2001). HIV replicating in an anergic, activated immune environment increases viral burden, while competent antiviral CTL responses are attenuated.

Furthermore, the β–chemokines (natural ligands of the chemokine receptor, CCR5), macrophage inflammatory protein 1α (MIP) 1-α, MIP1- β and RANTES have been repeatedly shown to have suppressive anti-HIV activity by different mechanisms (Cocchi *et al*., 1995; Amella *et al*., 2005). Decreased expression of these β–chemokines as a result of chronic infection with helminths, is documented (Kalinkovinch *et al*., 1999; Borkow *et al*., 2000; Gopinath *et al*., 2000).

From the above data, it is clear that HIV replication is more likely to be enhanced by helminth-induced anergy, attenuated control of replication and decreased antigen-specific CTL and antiviral activity. All these mechanisms create a conducive environment for HIV replication by attenuating innate and CTL responses against HIV.

1.6.4 Modulation of non-parasite responses by helminth infection.

As pointed out in the previous section above, helmiths induce anergy. The anergic responses initially are antigen–specific (Clerici and Shearer, 1993), downmodulating responses to the infecting parasite. However dysregulation of effective responses later extends to bystander cells, resulting in generalised reduction of responsiveness to mitogens, recall and other non-parasite antigens (Maizels and Yazdanbakhsh, 2003). The generalised immunosuppression is accompanied by accelerated apoptosis.

Additionally, the Th_2 predominance induced by helminthiasis also extends to uninfected cells such that naïve T cells expressing different repertoires of receptors are primed to differentiate to a Th_2 phenotype (Liu *et al*., 2002). Accordingly, suppression of Th_1 responses by helminth infection is well documented in both animals (Elias *et al.,* 2005) and humans (Cooper *et al*, 1998; 2000; 2001). For public health implications, several human studies have reported altered Th_1 responses to vaccines, which was attributed to immune modulation by helminth parasites. Albendazole pre-treatment of Ascaris infection improved antibody responses to cholera toxin in children infested by this parasite (Cooper *et al*., 2000). In young adults, the Albendazole-treated group had significant post-treatment increases in IFNγ responses to a recombinant cholera toxin and significantly higher IL-2 responses compared to the placebo group (Cooper *et al*., 2001). Similar trends of impaired Th_1 immune responses (IFNγ production) to tetanus toxoid-specific response in humans infected with Schistosoma (Sabin *et al*., 1996) and *Onchocerca volvulus* (Cooper *et al*., 1998) were demonstrated. Likewise, elimination of helminths by Albendazole treatment improved T cell proliferation and IFNγ production by PBMC's as well as PPD-specific responses in BCG-vaccinated individuals (Elias *et al*., 2001).

Furthermore, an extensive prospective study monitoring BCG vaccine efficacy from birth to 14 years of age in infants and children born of schistosomiasis- and filariasis- infected mothers was undertaken in Kenya where both parasites are endemic (Malhotra *et al.,* 1999). This study showed that *in utero*- sensitized infants acquire parasite immunity which lasts for up to 10-14 months after birth. IFNγ production in response to BCG vaccination by T cells of these sensitized infants was 26-fold lower than for children not sentitised to filariasis and schistosomiasis during gestation. Moreover, T cells from 2-to 10- year old children of mothers without parasite infection produced 10 fold more IFNγ in response to mycobacterial purified protein derivative (Malhotra *et al*., 1999). In 2005 Elias and co-workers also demonstrated an attenuated response to BCG in mice with schistosomiasis. Higher colony-forming units of TB bacilli, reduction in lung air exchange area and significantly less IFNγ production by splenocytes was found in these mice relative to their uninfected counterparts.

These attenuated responses to vaccines have serious complications for HIV vaccine development in areas where helminth infections are prolific.

1.7 Regulatory T cells in HIV and in helminth infections

The role of regulatory T cells (T_{reg}) in both parasitic helminth and HIV infections has been reviewed in detail and in both infections, their expression follows the activation of the immune system as a downmodulatory mechanism to control possible immunopathological sequelae of uncontrolled activation (Borkow and Bentwich, 2004). Their induction and activity in one infection may create either a favourable or an inimical environment for the establishment and development of the other. For example it is suggested that the decrease in T_{reg} during the chronic stages of HIV may disrupt the balance between activation and suppression of the immune system that is required for a synergistic co-existence of nematode parasites within the host (Brown *et al*, 2006). On the other hand, upregulation of these cells by helminths may counter the proinflammatory processes that are essential for the transcription and replication of the retroviruses including HIV. The role of regulatory cells is still a subject of debate regarding the interaction between HIV and other diseases including parasites (Brown *et al*, 2006).

1.8 Human studies evaluating direct helminth-HIV immunological interactions

Data explored above is mainly hypothetical, although they form a strong supportive basis upon which helminth infections are implicated as co-factors in HIV pathogenesis. It would be difficult to directly evaluate this interaction in humans, therefore *in vitro* and *ex vivo* models have been used in studies that attempt to assess this interaction.

PBMCs from filaria-infected individuals were shown to be more susceptible to HIV infection (Gopinath *et al.*, 2000). In this study viral replication as measured by reverse transcriptase (RT) values were higher but not statistically significant among the infected compared to the uninfected group. Of importance was the finding that 1-2 years post treatment RT values were significantly lower than the baseline pre-treatment levels of the same individuals, implying that elimination of filariasis decreased the susceptibility to rapid HIV replication. A similar study by Shapira-Nahor *et al.* (1998) revealed similar findings of increased susceptibility to HIV infection by PBMCs of helminth infected, activated individuals.

These *in vitro* studies, together with the findings (cited above) that helminth-infected animals and humans had impaired responses to vaccines plus the reports that deworming reversed the immune dysregulation observed in the immigrant Ethiopians (Bentwich *et al*., 1996; 1997; Kalinkovinch *et al*., 1998) lend strong support for a negative impact of helminth infections in HIV susceptibility. Nevertheless, human studies addressing the effect of eradication of helminth infections on HIV disease progression in different geographic localities have yielded inconsistent results, and sometimes suggesting a beneficial response of co-infection.

For example, Wolday *et al*., 2002 investigated the effect of helminth treatment on HIV progression through measurement of baseline and 6-month post treatment follow up of $CD4^+$counts and viral load among helminth-HIV coinfected Ethiopian participants. Stool examination of the 56 participants revealed 45% *Trichuris trichiura*, 41% *Ascaris lumbricoides*, 12% S*chistosoma* and 6% *Strongyloides* prevalence. In their findings, baseline viral loads and CD4 counts were similar between 31 helminth infected and 25 uninfected participants, however, a strong statistical difference between helminth intensity and viral load was found, with the heavier egg load associated with higher virus load. After 6 months follow up, there was a mean decrease of -0,36 $\log_{10}$ in HIV load among 13 successfully-treated participants compared to six who were persistently infected after treatment and nine who had been negative throughout the study period. In both the persistently infected and persistently negative participants, an increase in viral load was observed.

In Kenya, Lawn *et al.* (2000) compared viral loads before and 1-15 months after treatment of schistosomiasis among 30 male car washers. Stools and circulating anodic antigen (CAA) ELISA were used to confirm schistosomiasis. These authors did not find a correlation between eradication of schistosoma infection and reduction in HIV load. Instead, a post treatment-interval-dependent increase in viral load was found.

However, in another eport, Kallestrup and coworkers (2005) reported a positive impact of treatment of schistosomiasis among HIV-schistosomiasis co-infected Zimbabweans.
Their controlled cohort was randomised into early (baseline, n=64) or delayed (3 month, n=66) praziquantel intervention groups with or without (n=97) HIV-1 co-infection. Stool, urine and CAA were used in the diagnosis of schistosoma infection. Early treatment of

schistosomiasis was associated with a significant increase in CD4 counts, and arrested increase in viral load, while there was an increase in HIV RNA load in the delayed-treatment group. No changes were observed in CD4 counts among the HIV negative controls. An important finding of this study was an association between schistosomiasis co-infection and amplified HIV replication.

In Zambia (Modjarrad *et al.,* 2005) no significant association between treatment of helminths and reduction of viral load was found. At baseline, the viral load was lower among the helminth infected participants and a higher median CD4 count was observed throughout the study period in this group. In both helminth co-infected (n=54) and uninfected (n=57) groups, a slight increase in viral load and slight decreases in CD4 counts were noted four months after treatment for helminths which were predominantly roundworm (48%),hookworm (33%),schistosomiasis (9%) and other nematodes were less than 5%.

In Uganda, Elliot *et al*. (2003) compared 34 helminth-infected and 34 uninfected HIV-1 positive, ART-naive individuals to evaluate the effects of co-infection. The most common helminths found in this cohort were schistosoma and hookworm species, diagnosed by eggs in stools. At baseline, helminth-infected individuals had slightly lower viral load, significantly higher CD4 counts and CD4:CD8 ratio. Among the helminth uninfected group there was a progressive decline in $CD4^+$ counts from baseline to five weeks and four months follow up while no significant change in $CD4^+$ was found in those who were treated for helminthiasis. No significant change was found in viral load between baseline and four months post treatment in both groups. However, five weeks after treatment for helminths, a transient increase in viral load was observed in all groups, which was followed by a significant decline at four months among schistosomiasis-treated individuals. These authors concluded that their data does not support the notion that helminth infection worsens HIV infection, instead they suggest a possibility that helminthiasis may hinder HIV replication.

Again in Uganda**,** Brown *et al.* (2004) studied the association between helminthiasis and HIV-1 progression in a larger cohort (163 participants). Schistosoma and hookworm infections were the most common. They complemented stool screening with serological assays for schistosomiasis CAA ELISA; charcoal culture for strongyloides and Knotts

concentration method for microfilaria. Similarly, they reported no association between helminth infection and lower CD4 counts, higher viral load and faster decline in $CD4^+$ counts. In fact helminth infected participants were reported to have higher CD4 counts than their non infected counterparts, and a significantly higher median $CD4^+$ count was found in participants with hookworm infection. There were no significant changes in the viral load after treatment (except in *Mansonella perstans* infected participants in whom failure to clear the infection was associated with a decrease in viral load). The authors concluded that treatment of helminth infections may not be beneficial in slowing HIV replication (Brown *et al*., 2004).

In Malawi, neither helminth parasitic infection nor treatment thereof had an impact on HIV viral load in a cohort of 389 participants with prevalence of 43% helminths and 68% HIV (Hosseinipour *et al*., 2007).

As can be noted, two of the seven cited studies reported a beneficial effect of deworming in HIV–helminth co-infected participants (Wolday et *al*., 2002; Kallestrup *et al*., 2005). The rest did not find this effect, instead some even reported an increase in viral load after treatment (Lawn *et al*., 2000; Elliot *et al*., 2003; Brown *et al.,* 2004; Modjarrad *et al.,* 2005). There may be several possible factors contributing to these different findings:

- In both studies in Uganda (Elliot *et al*., 2003; Brown *et al*., 2004), *Schistosoma mansoni* was most prevalent; whereas in the Ethiopian (Wolday *et al*., 2002) and Zambian studies (Modjarrad *et al.,* 2005) the other intestinal parasites were more common. This would concur with the suggestion by Modjarrad and colleagues (2005) that immunological influences exerted by the tissue-resident schistosoma may differ from those of surface dwelling intestinal nematodes. However, both Lawn *et al.* (2003) and Kallestrup *et al.* (2005) investigated schistosomiasis and HIV co-infections and found inconsistent results. Furthermore, Modjarrad and colleagues' (2005) findings differed from those reported by Wolday *et al*. (2002) despite the predominance of intestinal parasites in both studies.

- In some of the studies efforts were made to increase the sensitivity of parasite diagnosis, such as serology for schistosomiasis, special concentration for

microfilaria and charcoal culture for strongyloides. However, diagnosis of the intestinal nematodes, particularly the most common (*Ascaris lumbricoides* and *Trichuris trichuria*) depended solely on detection of eggs from stools in all the studies (some on single collection). This method is known to have limited diagnostic value and therefore the error of misclassifying these infections as a result of false negatives would invariably be high. Wolday *et al*., 2002 collected two samples 4 hours apart within the same day, but the day-to-day variation in egg excretion would still not be overcome.

- Differences in study designs may also be a contributing factor. Some were double blind placebo controlled, and others were not blinded, while some compared the effect of treatment by comparing the impact of successful treatment versus persistent infection after treatment. Some of these studies lacked appropriate HIV uninfected comparison groups (Modjarrad *et al.,* 2005).

- The cohort sizes were markedly varied.

- Types of helminths and intensity of infestation may be another important factor. Wolday *et al*. (2002) and Modjarrad *et al.* (2005) reported significant association between helminth load and viral load, with the higher helminth load corresponding to higher viral RNA. Therefore differences in study outcomes might have been influenced by differences in intensities and types of helmiths in the different studies.

- The time interval between treatment and follow up may possibly have influenced some of these differences. It has been suggested that the immunological influence exhibited by schistosomiasis may be different from the superficial intestinal nematodes, and the time interval to have noticeable immunological effects may subsequently differ (Modjarrad *et al.,* 2005).

Other sources of bias:

1. **Inter-individual variation** in the primary outcomes (viral load and CD4 counts). These parameters vary greatly from individual to individual. Some studies compared changes in these two factors between groups who were and were not infected by helminths, or between those who were successfully treated and those persistently infected at follow up, or between early and delayed intervention groups, undermining the possible confounding by inter-individual variation. Four of the studies (Wolday *et al.*,2002; Brown *et al.*,2004; Elliot *et al.*,2003; Modjarrad *et al.*,2005) compared data of the same individuals at baseline and at follow up after treatment. These were the strenthgs of these studies.

2. **The possible immunomodulatory** effect of anthelminthic treatment was not considered in some of the studies. In only two of the studies (Wolday *et al.*, 2002; Brown *et al.*, 2004) were all the participants treated, including those who were not found to be infected by helminths. The rest of the authors reported treatment of those found to be infected by helminths only.

3. **The lack of blinding to intervention**. Kallestrup *et al.*(2005) assigned schistosoma infected individuals to early and delayed treatment arms Hence in this study there was no blinding.

4. Measurement of successful treatment by clearance of stool eggs could be another source of confounding, where false negatives coul be classified as successfully treated participants who were then compared to persistently-infected individuals.

The strengths and weaknesses identified in the studies:

The strengths of the study by Kallestrup *et al.* (2005) was the randomised, controlled trial design, however the weakness was the unblinded assignment to early and delayed treatment arms.

Modjarrad *et al.*,(2005) matched the 54 HIV and helminth infected participants for age and sex, and this was a strength of the study, however, not all participants received anthelminth treatment, which weakened the investigation.

In summary, the significant reduction of activated $CD4^+$ and $CD8^+$ T cells in HIV-1 negative Ethiopian adults following treatment of helminth infection (Kassu *et al.*, 2003), the reduction of immune activation by the immunosuppressant cylosporin A during ART initiation which is associated with improved $CD4^+$ counts (Rizzardi *et al.*, 2002) and the *in vitro* studies cited above in section 4 all lend strong support for the notion of increased susceptibility to HIV infection by concurrent helminth infection. However, there have been conflicting results from other studies and the effect of helminth infection severity, helminth types or other confounding and as yet unexplained mechanisms on HIV pathogenesis and HIV infection outcome remain to be elucidated. Human *in vivo* studies that attempt to determine the effect of infection by and treatment of parasites need to be standardised, be undertaken in different geographic locations in order to provide comparable and compelling evidence for the role played by helminths on HIV pathogenesis.

1.9 Study hypothesis

Individuals co-infected with HIV and intestinal helminths have impaired immune responses to HIV.

Some of the key aspects that are proposed to play a role in this interaction will be evaluated in the current work. These include the suggestions that:

- Helminth infections result in altered composition of peripheral blood lymphocyte populations.
- Helminth infections induce chronic immune activation.
- Chronic immune activation by helminths will increase viral load.
- Helminthiasis results in a predominance of Th_2 cytokines which down modulate Th_1 cytokines that are essential for control of viral infections.
- Helminth infection results in specific and generalised T cell anergy.

1.10 Study aim

The study intends to characterize the immune profiles of South African HIV-1 sero-positive and sero-negative individuals living in a helminth-endemic area and correlate these with HIV immune responses.

1.11. Objectives

1. To characterise the peripheral blood lymphocyte phenotypes of HIV infected and uninfected individuals with or without intestinal helminth co-infection.
2. To determine the activation status of lymphocytes in the participant subgroups with single or dual infections.
3. To assess functional characteristics (cytokine and lymphoproliferative responses) of lymphocytes in participants with and without HIV / intestinal helminths co-infection.
4. To correlate the above findings with immune responses to HIV.

1.12 Outline of the thesis

In Chapter 1 the literature was reviewed and Chapter 2 describes the study design, the preliminary tests that were done mainly by the supporting laboratories and the baseline characteristics of the participants. Chapter 3 defines the lymphocyte phenotyes of all suitable participants with complete data sets. In the subsequent Chapters 4-5, a subset of the participants was selected for analysis of immune activation, cytokine and lymphoproliferative responses to stimulation. Owing to the costs of analytic tests, selection requirements for the tests and budgetary constraints, only a limited number of samples could be investigated. The selected samples had to have enough cells (at least 5.5×10^6 cells/ml) in order to analyse the various immune components. Furthermore, participants with infections other than HIV and helminths had to be excluded. Therefore, for these analyses, a subgroup of sixty two participants (40 HIV^+ and 22 HIV^-) who did not have TB or sexually transmitted infections (STI) was selected. Their PBMC's were examined for activation, proliferation and cytokine production in response to antigen

stimulation. Chapter 6 summarises the study challenges and limitations that had been described in each relevant chapter. Chapter 7 discusses all the results and draws conclusions and provides recommendations.

References

Adams VJ, Markus MB, Kwitshana ZL, *et al*. 2006. Recall of intestinal helminthiasis by HIV-infected South Africans and avoidance of possible misinterpretation of egg excretion in helminth/HIV co-infection analyses. *BMC Infectious Diseases.* **6 (88):** 1-8.

Addo MM, Draenert R, Rathod A, *et al*. 2007. Fully differentiated HIV-1 specific CD8$^+$ T effector cells are more frequently detectable in controlled than in progressive HIV-1 infection. **www.plosone.org.** *Plos one* **(3)** e321. 1-7**.**

Allen JE, Maizels RM. 1997. Th1-Th2: reliable paradigm or dangerous dogma? *Immunology Today* **18:** 387-392**.**

Amella CA, Sherry B, Shepp DH. 2005. Macrophage inflammatory protein 1α inhibits postentry steps of Human immunodeficiency virus type 1 infection via suppression of intracellular cyclic AMP. *Journal of Virology***. 79 (9):** 5625-5631.

Ancuta P, Kamat A, Kunstman KJ *et al*. 2008. Microbial translocation is associated with increased monocyte activation and dementia in AIDS patients. PLoS ONE 3(6): e2516. doi:10.1371/journal.pone.0002516. Accessed 30-10-2010.

Bachou H , Tylleskär T , Downing R, Tumwine JK. 2006. Severe malnutrition with and without HIV-1 infection in hospitalised children in Kampala, Uganda: differences in clinical features, haematological findings and CD4+ cell counts. *Nutrition Journal.* **5**:27doi:10.1186/1475-2891-5-27

Badley AD, Pilon AA, Landay A, Lynch DH. 2000. Mechanisms of HIV-associated lymphocyte apoptosis. *Blood***. 96:** 2951-2964.

Bahbouhi B, Landay A, Al-Harthi L. 2004. Dynamics of cytokine expression in HIV productively infected primary CD4$^+$ T cells. *Blood.* **103 (12):**4581-4587

Bailey JR, Lassen KG, Yang HC, *et al*. 2006. Neutralising antibodies do not mediate suppression of Human Immunodeficiency Virus Type 1 in Elite suppressors or selection of plasma virus variants in patients on Highly Active Antiretroviral Therapy. *Journal of Virology.* **80 (10):** 4758-4700.

Barsoum IS, Freeman GL jr., Habib M, *et al.* 1984. Evaluation of Natural Killer activity in human schistosomiasis. *American Journal of Tropical Medicine and Hygiene.* **33 (3):** 451-454.

Bentwich Z, Kalinkovich A, Weisman Z. 1995. Immune activation is a dominant factor in the pathogenesis of African AIDS. *Immunology Today.**16 (4):*** 187-191.

Bentwich Z, Kalinkovich A, Weisman Z, *et al.* 1999. Can eradication of helminthic infections change the face of AIDS and tuberculosis? *Immunology Today.**20*** (11): 485-487.

Bentwich Z, Maartens G, Torten D, Lal A A, Lal RB. 2000. Concurrent infections and HIV pathogenesis. *AIDS*. **14 (14) :** 2071-2081

Bentwich Z, Weisman Z, Moroz C, Bar-Yehuda S, Kalinkovich A. 1996. Immune dysregulation in Ethiopian immigrants in Israel: relevance to helminth infections? *Clinical Experimental Immunology.* **103:** 239-243.

Bentwich Z, Weisman Z, Grossman Z, Galai N, Kalinkovich A. 1997. Pathogenesis of AIDS in Africa - lessons from the Ethiopian immigrants in Israel. *Immunologist.* **5:** 21-26.

Beretta A, Weiss SH, Rappocciolo G**,** *et al*. 1996. Human immunodeficiency virus type 1 (HIV-1)-seronegative injection drug users at high risk for HIV exposure have antibodies to HLA class 1 antigens and T cells pecific for HIV envelope. *Journal of Infectious Diseases.* **173:** 472-476.

Betts MR, Ambrozak DR, Douek DC, *et al.* 2001. Analysis of total human immunodeficiency virus (HIV)-specific $CD4^+$ and $CD8^+$ T–cell responses: Relationship to viral load in untreated HIV infection. *Journal of Virology*. **75 (24):**11983-11991.

Betts CJ, deSchoolmeester ML, Else KJ. 2000. Trichuris muris: $CD4^+$ T cell-mediated protection in reconstituted SCID mice. *Parasitology.* **121:**631-637.

Betts MR, Nason MC, West SM, *et al.* 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8$^+$ T cells. *Blood.* **(107) 12:** 4781-4789.

Blanco J, Barretina J, Clotet B, Este JA. 2004. R5 HIV gp120-mediated cellular contacts induce the death of single CCR5-expressing CD4T cells by a gp41-dependent mechanism. *Journal of Leukocyte Biology*. **76:** 804-811.

Bleul CC, Wu L, Hoxie JA, *et al.* 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proceedings of the National Academy of Science USA*. **94:**1925-1930.

Borkow G, Leng Q, Weisman Z, *et al.* 2000. Chronic immune activation associated with intestinal helminth infections results in impaired signal transduction and anergy. *Journal of Clinical Investigation*. **106 (8):** 1053–1060.

Borkow G, Bentwich Z. 2004. Chronic immune activation associated with chronic helminthic and Human Immunodeficency Virus infections: Role of hyporesponsiveness and anergy. *Clinical Microbiology Reviews.* **17(4):** 1012-1030.

Boritz E, Palmer BE, Wilson CC. 2004. Human immunodeficiency virus type1 (HIV-1)-specific CD4$^+$ T cells that proliferate *in vitro* detected in samples from most viraemic participants and inversely associated with plasma HIV-1 levels. *Journal of Virology*. **78(22):**12638-12646.

Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MBA. 1994. Virus-specific CD8$^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *Journal of Virology.* **68**: 6103−6110

Borrow P, Lewicki H, Wei X, *et al.* 1997. Antiviral pressure exerted by HIV-l-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Medicine.* **3:** 205 – 211.

Bour-Jordan H, Bluestone JA. 2002. CD28 function: A balance of costimulatory and regulatory signals. *Journal of Clinical Immunology*. **22 (1):**1-7.

Bradshaw D, Steyn K, editors. 2002. Poverty and Chronic Diseases in South Africa: *Technical Report 2001***.** *South African Medical Research Council.*

Brake D A. 2003. Parasites and Immune responses: Memory Illusion? *DNA and Cell Biology***. 22(6):** 405-419.

Braganza R, Beddows S, Frazeo S, *et al.* 2001. Genetic, virological and immunological characterization of a cohort of long-term monogamous HIV discordant couples. *8th Conference on Retroviral Opportunistic Infections. Sheraton Hotel Towers Chicago Ill***.** Feb 4-8; **8: 59** (abstract no. 52).

Brenner BG, Gornitsky M, Wainberg MA. 1994. Interleukin-2-inducible Natural immune (Lymphokine-Activated Killer Cell) responses as a functional correlate of progression to AIDS. *Clinical and Diagnostic Laboratory Immunology*. **1: (5)** 538-544.

Brenchley JM, Mirko P, Knox KS, *et al.* 2008. Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections. *Blood.* **112(7)**: 2826–2835

Brodskyn CI, DeKrey GK, Titus RG. 2001. Influence of costimulatory molecules on immune response to *Leishmania major* by human cells *in vitro. Infection and Immunity.***69 (2):** 665-672.

Brown DR, Moskowitz NH, Nigel Killeen N, Reiner SL. 1997. A role for CD4 in peripheral T cell differentiation. *The Journal of Experimental Medicine. (***186) 1:** 101-107.

Brown M, Kizza M, Watera C, *et al.* 2004. Helminth infection is not associated with faster progression of HIV disease in coinfected adults in Uganda. *The Journal of Infectious Diseases.* **190:**1869-1879.

Bundy, D., Sher, A., Michael, E., 2000. Good worms or bad worms: do worm infections affect the epidemiological patterns of other diseases? ***Parasitol. Today* 16, 273-274**.

Burastero S, Casoli C, Paoluco C, *et al.* 2006**.** Anti-CD4-gp120 complex antibodies in long term non-progressors HIV-1 positive patients: a role in disease progression? *Retrovirology*. **(3) (Suppl 1)** 36.

Callaway DS, Ribeiro RM, Nowak MA. 1999. Virus phenotype switching and disease progression in HIV-1 infection. *Proceedings of the Royal Society of London: Biological Sciences.* **266 (1437):** 2523-2530.

Campbell KA, Ovendale PJ, Kennedy MK, Fanslow WC, Reed SG, Maliszewki CR. 1996. CD40 ligand is required for protective cell-mediated immunity to Leishmania major. *Immunity.* **4(3)**: 283-289.

Carmichael A, Jin X, Sissons P, Borysiewicz L. 1993. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. *Journal of Experimental Medicine*. **177:**249-256.
Caruso C , Buffa S, Candore G, Colonna-Romano G , Dunn-Walters D, Kipling D, Pawelec G. 2009. Mechanisms of immunosenescence. *Immunity & Ageing* **6:**10doi:10.1186/1742-4933-6-10. Accesses 02 11 2010.

Castedo M, Perfettini J-L, Andreau K. *et al*. 2003. Mitochondrial apoptosis induced by the HIV-1 envelope. *Annals of the New York Academy of Sciences*. **1010:** 19-28.

Chen S, Tuttle DL, Oshier JT, *et al*. 2005. Transforming growth factor-1β increases CXCR4 expression, stromal derived factor 1α-stimulated signalling and human immunodeficiency virus-1 entry in human monocyte-derived macrophages. *Immunology.* **114.** 565-574.

Chien PC jnr, Chen D, Chen P-d, *et al*. 2004. HIV-1- infected patients with envelope-specific lymphoproliferation or long term non-progression lack antibodies suppressing glycoprotein 120 antigen presentation. *Journal of infectious Diseases.* **189:**852-861**.**

Ciurea A, Klenerman P, Hunziker L, *et al.* 2000. Viral persistence *in vivo* through selection of neutralizing antibody-escape variants. *Proceedings of the National Academy of Sciences of the USA*. **97 (5):**2749-2754.

Clerici M, Declich S, Rizzardini G. 2001. African enigma: key player in Human Immunodeficiency Virus pathogenesis in developing countries? *Clinical and Diagnostic Laboratory Immunology.* **8(5):**864-866.

Clerici M, Sarin A, Berzosky JA, *et al.* 1996. Antigen-stimulated apoptotic T cell death in HIV infection is selective for $CD4^+$ cells, modulated by cytokines and effected by lymphotoxin. *AIDS*.**10:** 603-611.

Clerici M, Sarin A, Coffman RL, *et al.* 1994. Type 1/type2 cytokine modulation of T-cell programmed cell death as a model of human immunodefficiency virus pathogenesis. *Proceedings of the National Academy of Sciences of the USA.* **91:**11811-11815**.**

Clerici M, Shearer GM. 1993. A TH1→TH2 switch is a critical step in the etiology of HIV infection. *Immunology Today.* **14 (3):**107 - 111.

Clerici M, Stefano B, Matthew L *et al.* 2000. Immune activation in Africa is enviromentally-driven and is associated with upregulation of CCR5. *AIDS* **14 (14):** 2083-2092.

Clouse KA, Cosentino LM, Weih KA, *et al.* 1991. The HIV-1 gp120 envelope protein has the intrinsic capacity to stimulate monokine secretion. *The Journal of Immunology,* **147(9):** 2892-2901.

Cocchi F, De Vico AL, Garzino-Demo A, *et al.* 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by $CD8^+$ T cells. *Science.* **270:**1811-1815.

Connor RI, Mohri H, Cao Y, Ho DD. 1993. Increased viral burden and cytopathicity correlate temporally with $CD4^+$ T-lymphocyte decline and clinical progression in human

immunodeficiency virus type 1-infected individuals. *Journal of Virology*. **67(4):** 1772–1777.

Conway B, Baskar P, Bechtel LJ, *et al* .1992. Eosinophils as host cells for HIV-1. *Archives of Virology*.**127 (1) 4:**373-377.

Cooper PJ, Chico ME, Losonsky G, *et al.* 2000. Albendazole treatment of children with ascariasis enhances the vibriocidal antibody response to the live attenuated oral cholera vaccine CVD 103-HgR. *The Journal of Infectious Diseases.* **182:** 1199-1206.

Cooper PJ, Chico ME, Sandoval C, *et al.* 2001. Human infection with *Ascaris lumbricoides* is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. *Infection and Immunity.* **69(3):** 1574-1580.

Cooper PJ, Espinel I, Parades W, *et al.* 1998. Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocerciais: a possible role for interleukin -10. *The Journal of Infectious Diseases.* **178(4):** 11 33-1138.

Cottrezz F, Manca F, Dalgeish AG, *et al*. 1997. Priming of human $CD4^+$ antigen-specific T cells to undergo apoptosis by HIV-infected monocytes. *The Journal of Clinical Investigation.* **99:** 257-266.

Cunningham-Rundles S, McNeeley DF, Moon A. 2005. Mechanisms of nutrient modulation of the immune response. *The Journal of Allergy and Clinical Immunology*.**115** (6):1235-1237.

Day CL, Kiepiela P, Leslie AJ, *et al.* 2007. Proliferative capacity of epitope-specific CD8 T–cell responses is inversely related to viral load in chronic human immunodeficiency virus type 1 infection. *Journal of Virology*. **81 (1):** 434-438.

De Boer RJ, Mohri H, Ho DD, Perelson AS. 2003. Turnover rates of B cells, T cells and NK cells in Simian Immunodeficiency vurus –infected and uninfected Rhesus Macaques**.** *The Journal of Immunology.* **170:**2479-2487.

De Milito A. 2004. B lymphocyte dysfunction in HIV infection. *Current HIV Research*. **2:** 11-21.

De Milito A, Nilsson A, Titanji K, *et al*. 2004. Mechanisms of hypergammaglobulinemia and impaired antigen-specific humoral immunity in HIV-1 infection. *Blood.* **103:** 2180-2186

De Souza MS, Karnasuta C, Brown AE, *et al.* 2000. A comparative study of the impact of HIV on Natural Killer cell number and function in Thais and North Americans. *AIDS Research and Human Retroviruses.***16: (11)** 1061-1066.

Diaz A, Allen JE. 2007. Mapping immune response profiles: The emerging scenario from helminth immunology. *European Journal of Immunology.* **37:**1-8.

Douek CD. Disrupting T-cell homeostasis: how HIV-1 infection causes disease. 2003. *AIDS Reviews.* **5 (3):** 172-177.

Douek DC, Betts MR, Hill BJ, *et al*. 2001. Evidence for increased T cell turnover and decreased thymic output in HIV infection. *Journal of Immunology.* **167:** 6663–6668.

Drake L, Maier C, Jukes M, Patrikios A, 2002. School-age children: their nutrition and health. *SCN News: a periodic review of developments in international nutrition. United Nations System. Standing Committee on Nutrition*. **25:** 4-30.

Dunne DW, Butterworth AJ, Fulford JH. 1992. Human IgE responses to *Schistosoma mansoni* and resistance to reinfection. *Memorial Institute Oswaldo Cruz*. **87 (S4):**99-115.

Elias D, Akuffo H, Pawloski A, *et al*. 2005. *Schistosoma mansoni* infection reduces the protective efficacy of BCG vaccination against virulent *Mycobacterium tuberculosis. Vaccine.***23:**1326-1334.

Elias D, Wolday D, Akuffo H, *et al.* 2001. Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clinical Experimental Immunology.* **123:**219-225.

Elliot MA, Mawa PA, Joseph S, *et al.* 2003. Association between helminth infection and $CD4^+$ T cell count, viral load and cytokine responses in HIV-1 infected Ugandan adults.*Transactions of the Royal Society of Tropical Medicine and Hygiene.* **97:**103-108.

Else KJ, Finkelman FD. 1998. Intestinal nematode parasites, cytokines and effector mechanisms*. International Journal of Parasitology***. 28 (8):**1145-1158.

Else KJ, Grencis RK. 1996. Antibody-independent effector mechanisms in resistance to the intestinal nematode parasite *Trichuris muris*. *Infection and Immunity.* **64 (8):** 2950-2954.

Evans AC, du Preez L, van der Merve CA, Schutte CHJ. 1987. Observations on the helminth infections in black pupils of the Eastern Transvaal Lowveld of South Africa. *South African Journal of Epidemiology and Infection***. 2:**7-14.

Fauci AS. 1993. Multifactoral nature of Human Immunodeficiency Virus Diease. Implications for therapy. *Science.* **262:**1011-1018.

Faulkner H, Turner J, Kamgno J, *et al.* 2002. Age-and infection intensity-dependent cytokine and antibody production in human trichurriasis: the importance of IgE. *The Journal of Infectious Diseases.* **185:**665-672.

Feldmeier H, Gastl GA, Poggensee U, Kortmann C. 1985. Relationship between intensity of infection and immunomodulation in human schistosomiasis. II. Nk cell activity and *in vitro* lymphocyte proliferation. *Clinical Experimental Immunology.* **(60):** 234-240**.**

Ferland C, Flamand N, Davoine F, *et al.*, 2004. IL-16 activates plasminogen-plasmin system and promotes human eosinophil migration into extracellular matrix via CCR3-

chemokine mediated signalling and modulating CD4 eosinophil expression. *Journal of Immunology.* **173 (7):** 4417-4424.

Fincham J, Dhannsay A. 2006. Helminths in SA's children. *MRC Policy brief* **No. 2.**

Fincham JE, Evans AC, Woodrof CW, *et al.* 1996. Feed the children, not the parasites – an essential part of primary health care in South Africa, *South African Medical Journal***.** **86(6):** 647-649.

Fincham JE, Markus MB, Adams VJ, *et al.*, 2003. Association of deworming with reduced eosinophilia: implications for HIV/AIDS and co-endemic diseases. *South African Journal of Science.* **99:**182-184.

Fontenot JD, Gavin MA, Rudensky AY. 2003 Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. *Nature Immunology*. **4**:330–336.

Fowell DJ, Magram J, Turck CW, *et al.* 1997**.** Impaired Th_2 subset development in the absence of CD4*.* *Immunity.* **6(5):**559-569.

Freedman AR, Gibson FM, Fleming SC, *et al.* 1991. Human immunodeficiency virus infection of eosinophils in human bone marrow cultures. *The Journal of Experimental Medicine***.** **174:**1661-1664.

Gajewski TF, Pinnas M, Wong T, Fitch FW. 1991. Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *The Journal of Immunology.* **146: (6)** 1750-1758.

Geiger SM, Massara CL, Bethony J, *et al.* 2002. Cellular and cytokine profiles in *Ascaris lumbricoides* and *Trichuris trichiura* infected patients. *Parasite Immunology***.24:** 499-509**.**

Giorgi JV, Hultiri LE, McKeating JA, *et al.* 1999. Shorter survival in advanced human immunodeficiency virus type 1 inffection is more closely associated with T lymphocyte activation than with plasma virus burden or virus coreceptor usage. *The Journal of Infectious Diseases.* **179 (4):**859-870.

Gopinath R, Ostrowski M, Justement SJ *et al.* 2000. Filarial infections increase susceptibility to Human Immunodeficiency virus infection in peripheral blood mononuclear cells *in vitro*. *The Journal of Infectious diseases.* **182:**1804-1808.

Gougeon ML, Montagnier L.1993. Apoptosis in AIDS.*Science.* **260:**1269-1270.

Gougeon ML. 2003. Cell death and Immunity. Apoptosis as an HIV strategy to escape immune attack. *Nature Reviews Immunology***. 3:** 392- 404.

Graziosi C, Pantaleo G, Gantt KR, *et al.* 1994. Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals. *Science.* **265 (5169):**248-252

Grencis RK, Bancroft AJ. 2004. Interleukin-13. A key mediator in resistance to gastrointestinal- dwelling nematode parasites. *Clinical Reviews in Allergy and Immunology.* **26:** 51-60.

Grossman Z, Meier-Schellersheim M, Paul WE, Picker LJ. 2006. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nature Medicine***.12(3):**289-95.

Grunewald, Burmester, Schuler-Maue, *et al.* 1999. Anti-phospholipid antibodies and $CD5^+$ B cells in HIV infection. *Clinical and Experimental Immunology***. 115 (3):**464-471**.**

Hagel I, Lynch NR, Puccio F, *et al.* 2003. Defective regulation of the protective IgE response against intestinal helminth *Ascaris lumbricio*des in malnourished children. *Journal of Tropical Paediatrics***. 49 (3):**136-142**.**

Hart M, Steel A, Clark SA, *et al.* 2007. Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease. *Journal of Immunology***. 178**: 8212-8220.

Heinzel FP, Rerko RM, Hujer AM. 1998. Underproduction of interleukin-12 in susceptible mice during progressive leishmaniasis is due to decreased CD40 activity. *Cellular Immunology*. **184 (2):** 129-142.

Henri S, Chevillard C, Mergani A*, et al***.** 2002. Cytokine regulation of periportal fibrosis in humans infected with Schistosoma mansoni: IFN-γ is associated with protection against fibrosis and TNF-α with aggravation of disease. *The Journal of Immunology*. **169:** 929-936.

Ho J, Moir S, Malaspina A , *et al.* 2006. Two overrepresented B cell populations in HIV-infected individuals undergo apoptosis by different mechanisms. *Proceedings of the National Academy of Sciences of the USA.* **103:** 19436-19441.

Homsy J, Meyer M, Tateno M, Clarkson S, Levy JA. 1989. The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells. *Science*. **(244) 4910**: 1357-1360.

Hosseinipour M, Napravnik S, Joaki G, *et al.* 2007**.** HIV and parasitic infection and the effect of treatment among adult outpatients in Malawi. *The Journal of Infectious Diseases.* **195:** 1278-1282**.**

Hu PF, Hultin LE, Hultin P, *et al.*, 1995. Natural killer cell immunodeficiency in HIV is manifest by profoundly decreased numbers of $CD16^{+}CD56^{+}$ cells and expansion of a population of CD16 dim CD56- cells with low lytic activity. *Journal of Acquired Immunodeficiency Syndrome and Human Retroviruses.* **(10) 3:** 331-340.

Hunt PW. 2007. Role of immune activation in HIV pathogenesis.*Current HIV/AIDS Reports.* **4: (1)** 42-47.

Imami N, Pires A, Hardy G, *et al.* 2002. A balanced type1/type2 response is associated with long term nonprogressive Human Immunodeficiency Virus type 1 infection. *Journal of Virology.* **76 (18):**9011-9023.

Jackson JA, Turner JD, Rantoul L, *et al.*, 2004. Cytokine response profiles predict species-specific infection patterns in human GI nematodes. *International Journal for Parasitology* **34 (11):** 1237-1244.

Janeway CA, Travers P, Walport M, Shlomchik M. 2001**.** *In:* Schanck D, Austin P, Lawrence E, *et al.* eds.Antigen presentation to lymphocytes. *Immunobiology . The immune system in health and disease. 5th ed. Chapters 1-14. T-cell mediated immunity***.** Garland Publishing. Taylor and Francis group.

Jin X, Bauer DE, Tuttleton SE *et al.* 1999. Dramatic rise in plasma viremia after CD8$^+$ Tcell depletion in Simian Immunodeficiency virus-infected macaques**.** *Journal of Experimental Medicine.* **189 (6) :**991-998.

Jordan G, Frederich 1997. Cytokines and lymphocytes. In: Remick G and Friedland JS, Eds. Cytokines in Health and Disease. Chapter 24. ISBN0-8247-9823-6.

Kalams SA, Walker BD. 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *Journal of Experimental Medicine.* **188:** 2199.

Kalinkovinch A, Weisman Z, Greenberg Z, *et al.* 1998. Decreased CD4 and increased CD8 counts with T cell activation is associated with chronic helminth infection. *Clinical and Experimental Immunology.* **114**:414-421.

Kalinkovinch A, Weisman Z, Leng Q, *et al.* 1999. Increased CCR5 expression with decreased beta chemokine secretion in Ethiopians/: relevance to AIDS in Africa. *Journal of Human Virology***. 2 (5): 283-289.**

Kalinkovich A, Borkow G, Weisman Z**,** *et al.* 2001. Increased CCR5 and CXCR4 expression in Ethiopians living in Israel: environmental and constitutive factors. *Clinical Immunology.* **100:**107-117.

Kallestrup P, Zinyama R, Gomo E, *et al.* 2005. Schistosomiasis and HIV-1 infection in rural Zimbabwe: Effect of treatment of schistosomiasis on CD4 cell count and plasma HIV-1 RNA load. *The Journal of Infectious Diseases.* **192 (11):** 1956-1961.

Kassu A, Tsegaye A, Wolday D, *et al.* 2003. Role of incidental and/or cured intestinal parasitic infections on profile of CD4$^+$ and CD8$^+$ T cell subsets and activation status in

HIV-1 infected and uninfected adult Ethiopians. *Clinical Experimental Immunology. 132*:113-119.

Khoury SJ, Gallon L, Verburg R R, *et al.* 1996. *Ex vivo* treatment of antigen-presenting cells with CTLA-4 Ig and encephalitogenic peptide prevents experimental autoimmune encephalomyelitis in the Lewis rat. *Journal of Immunology*. **157:** 3700-3755.

Kiepiela P, Leslie AJ, Honeyborne I, *et al.* 2004. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature*. **432(9)** 769-775**.**

Klein MR, van Baalen CA, AM Holwerda AM, *et al.* 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *Journal of Experimental Medicine*. **181:** 1365-1372.

Klein SA, Dobmeyer JM, Dobmeyer TS, *et al.* 1997. Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokine detection on single cell level by flow cytometry*. AIDS.* **11(9):**1111-1118

Korten S, Volkmann L, Saeftel M, *et al*. 2002. Expansion of NK cells with reduction of their inhibitory Ly-49A, Ly-49C, and Ly-49G2 receptor-expressing subsets in a murine helminth infection: Contribution to parasite control*. The Journal of Immunology***. 168(10):**5199-5206.

Kottilil S, Shin K, Planta M, *et al*. 2004. Expression of chemokine and inhibitory receptors on Natural Killer cells: Effect of Immune activation and HIV viraemia*. The Journal of Infectious Diseases*. **189:**31193-1198.

Koup RA. 1994. Virus escape from CTL recognition. *Journal of Experimental Medicine.* **180 (3):** 779-782.

Koup RA, Safrit JT, Cao Y, *et al*. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary HIV-1 syndrome. *Journal of Virology***. 68:**4650-4655.

Kuchroo VK, Das MP, Brown JA, *et al.* 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 development pathways: application to autoimmune disease therapy. *Cell.* **80**: 707-718.

Kuhn L, Meddows-Taylor S, Gray G, Tiemessen C. 2002. Human Immunodeficiency Virus (HIV)–Specific Cellular Immune Responses in Newborns Exposed to HIV In Utero *Clinical Infectious Diseases***. 34:** 267–276.

Kurts C. 2007. Th17 cells: a third subset of $CD4^+$ T effector cells involved in organ-specific autoimmunity. *Nephrology Dialysis Transplantation* **23 (3):** 816-819.

Kwitshana ZL, Tsoka J,Mabaso MHL. 2008. Intestinal parasitic infections in adult patients in KwaZulu-Natal.*South African Medical Journal.***98** (9):709-711.

Lane HC, Masur H, Edgar LC, *et al.* 1983. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome***.*** *New Engand Journal of Medicine*. **309:**453-458.

Lawn SD, Butera S, Folks TM. 2001. The contribution of immune activation to the pathogenesis and transmission of Human Immunodeficiency Virus type1 infection. *Clinical Microbiology Reviews***. 14(4):** 753-777**.**

Lawn SD, Karanja DMS, Mwinzi P, *et al.* 2000. The effect of treatment of schistosomiasis on blood plasma HIV-1 RNA concentration in coinfected individuals.*AIDS.* **14:**2437-2443**.**

Leng Q, Borkow G,Weisman Z,*et al.* 2001. Immune activation correlates better than HIV plasma viral load with CD4 Tcell decline during HIV infection. *Journal of Acquired Immune deficiency Syndromes.* **27**:389-397.

Lertanekawattana S, Wichatrong T, Chaisari K, *et al.* 2005. Immunological characteristics of patients infected with common intestinal helminthes: results of a study

based on reverse-transcriptase PCR. *Annals of Tropical Medicine and Parasitology*. **99(1):**71-80.

Leslie AJ, Pfafferott KJ, Chetty P, *et al.* 2004. HIV evolution: CTL escape mutation and reversion after transmission. *Nature Medicine.* **(10) 3:** 282- 289.

Leutscher P, Ramarokoto CE, Reimert C, *et al*. 2000. Community-based study of genital schistosomiasis in men from Madagascar. *Lancet*. **355:**117-118.

Levy JA. 1993. Pathogenesis of Human Immunodeficiency Virus Infection. *Microbiological Reviews*. **57 (1):** 183-289

Liu Z, Liu Q, Pesce J, *et al*. 2002. *Nippostrongylus brasiliensis* can induce B7-independent antigen-specific development of IL-4 producing T cells from naïve $CD4^+$ T cells *in vivo. The Journal of Immunology.* **169:** 6959-6968.

Lopez-Balderas N, Huerta L, Villarreal C, *et al*. 2007. *In vitro* cell fusion between $CD4^+$ and HIV-1 Env^+ T cells generates a diversity of syncytia varying in total number, size and cellular content. *Virus Research*. **123:** 138-146.

Lucey DR, Dorsky DI, Nicholson-Weller A, Weller PF. 1989. Human eosinophils express CD4 protein and bind human immunodeficiency virus gp120. *The Journal of Experimental Medicine.***169.** 327-332.

Lynch NR, Hagel IA, Palenque ME *et al.* 1998. Relationship between helminthic infection and IgE response in atopic and nonatopic children in a tropical environment. *Journal of Allergy and Clinical Immunology.* **101(2 part 1):** 217-221.

Mabaso MLH, Appleton CC, Hughes JC, Gouws E. 2004. Hookworm (*Necator americanus*) transmission in inland areas of sandy soils in KwaZulu-Natal, South Africa. *Tropical Medicine &. International Health* **; 9 (4):**471-476.

Maek-A-Nantawat W, Buranapraditkun S, Klaewsongkram J, Ruxrungthum K. 2007. Increased Interleukin-17 production both in Helper T Cell subset Th17 and CD4-negative T cells in Human Immunodeficiency Virus Infection. *Viral Immunology*. **20(1**): 66-75.

Maggi E, Mazzetti M, Ravina A, *et al.*, 1994. Ability of HIV to promote a Th_1 to Th_0 shift and to replicate preferentially in Th_2 and Th_0 cells. *Science*. **265 (5169):**244-248.

Mahalingam M, Peakman M, Davies ET, Pozniak A, McManus TJ, Vergani D. 1993. T cell activation and disease severity in HIV infection. *Clinical Experimental Immunology*. 93(3): 337–343.

Maizels RM, Bundy DAP, Selkirk ME, Smith DF, Anderson RM. 1993. Immunological modulation and evasion by helminth parasites in human populations. *Nature* **365:** 797-805.

Maizels RM, Yazdanbakhsk M. 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms.*Nature Reviews Immunology***. 3:**733-744.

Maizels RM, Balic A, Gomez-Escobar N, *et al*. 2004. Helminth parasites- Masters of regulation.*Immunological Reviews.* **201:** 89-116.

Malhotra I, Mungai P, Wamachi A, *et al.* 1999. Helminth and Bacillus Calmette-Guérin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *The Journal of Immunology.* **162:** 6843-6848**.**

Masemola A, Mashishi T, Khoury G, *et al.* 2004. Hierarchical targeting of subtype C Human Immunodeficiency Virus type 1 proteins by $CD8^+$ T cells: Correlation with Viral load. *Journal of Virology.* **78(7):***3233-3243***.**

Mavillo D, Lombardo G, Benjamin J**,** *et al.* 2005. Characterisation of CD56-/$CD16^+$ natural killer cells: A highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proceedings of the National Academy of Sciences of the USA.* **102 (8)**:2886-2891.

Matloubian M, Concepcion RE, Ahmed R. 1994. $CD4^+$ T cells are required to sustain $CD8^+$ cytotoxic T cell responses during chronic viral infection. *Journal of Virology.* **68:**8056-8063**.**

McAdam AJ, Chang TT, Lumelsky AE, *et al.* 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of $CD4^+$ T cells. *The Journal of Immunology***. 165:**5035-5040**.**

McDermott JR, Grencis RK , Else KJ. 2001. Leucocyte recruitment during enteric nematode infection. *Immunology.* **103(4):** 505-510**.**

McDonald AS, Straw AD, Dalton NM, Pearce EJ. 2002. Cutting edge: Th2 response induction by dendritic cells: a role for CD40. *Journal of Immunology.* **168 :**537-540.

McKee AS, Pearce EJ. 2004. $CD25^+CD4^+$ cells contribute to Th2 polarisation during infection by suppressing Th1 response development. *The Journal of Immunology***.173:** 1224-1231.

McMichael AJ, Callan M, Appay V, Hanke T, Ogg G, Rowland-Jones S. 2000. The dynamics of the cellular immune response to HIV infection: implications for vaccination. *Philos Transactions of the Royal Society of London: Biological Sciences.* **355(1400):** 1007-1011.

Meier UC, Owen RE, Taylor E, *et al.* 2005. Shared alterations in NK cell frequency, phenotype and function in chronic human immunodeficiency virus and hepatitis C virus infections. *Journal of Virology***. 79 (19):**12365-12374**.**

Metzner KJ, Jin X, Lee VF, *et al.* 2000. Effects of *in vivo* $CD8^+$ T cell depletion on virus replication in Rhesus Macaques immunized with a live, attenuated Simian Immunodeficiency Virus vaccine. *Journal of Experimental Medicine.* **191 (11):**1921-1931.

Minami R, Yamamoto M, Takahama S, *et* al. 2006. RCSA 1 induced by HIV-Tat is involved in the apoptosis of HIV-1 infected and uninfected $CD4^+$ T cells. *Cellular Immunology.* **243:**41-47.

Moanna A, Dunham R, Paiardini M, Silvestri G. 1996. CD4 T cell depletion in HIV infection: killed by a friendly fire. *Current HIV/AIDS Reports* **2: (1)** 16-23.

Modjarrad K, Zulu I, Redden DT, *et al.* 2005. Treatment of intestinal helminths does not reduce plasma concentrations of HIV-1 RNA in coinfected Zambian adults. *The Journal of Infectious Diseases.* **192:**1277-1283.

Moir S, Malaspina A, Li Y, *et al.* 2000. B cells of HIV infected patients bind virions through CD21-complement interactions and transmit infectious virus to activated T cells. *The Journal of Experimental Medicine.* **192 (5):** 637-646.

Moir S, Malaspina A, Ogwaro KM, *et al.* 2001. HIV-1 induces phenotypic and functional pertubations of B cells in chronically infected individuals*. Proceedings of the National Academy of Sciences of the USA.* **10.1073/pnas:** 181347898

Montefiori DC, Hill TS, Vo HT, Walker BD, Rosenberg ES. 2001. Neutralizing antibodies associated with viremia control in a subset of individuals after treatment of acute human immunodeficiency virus type 1 infection. *Journal of Virology.* **75(21):**10200-10207.

Mosala TI. 2001. Geohelminth transmission among slum dwelling children in Durban, South Africa. *PhD Thesis***.** University of KwaZulu-Natal, Durban, South Africa.

Mosunjac MB, Tadros T, Beach R, Majmudar B. 2003. Cervical schistosomiasis, human papilloma virus (HPV), and human immunodeficiency virus (HIV): a dangerous coexistence or coincidence? *Gynaecological Oncology***. 90(1):** 211-214.

Myers B, Speight EL, Huissoon AP, Davies JM. 2000. Natural Killer cell lymphocytosis and strongyloides infection. *Clinical and Laboratory Haematology***. 22:** 237-238.

Niederkorn JY, Stewart GL, Ghazizadeh S, *et al.* 1988. *Trichinella pseudospiralis* larvae express Natural Killer (NK) cell-associated Asialo-GN1 antigen and stimulate pulmonary NK activity*. Infection and Immunity.* **55(5):**1011-1016**.**

Orenstein JM. 2000. *In Vivo* Cytolysis and Fusion of Human Immunodefivciency Virus Type-1 Infected Lymphocytes in Lymphoid Tissue. *The Journal of Infectious Diseases.* **182:**338-342.

Osborne J, Devaney E. 1998. The L3 of Brugia induces a Th_2 polarised response following activation of an IL-4 producing CD4-CD8- αβ T cell population. *Intestinal Immunology***.10:**1583-1590.

Patterson BK, Czerniewski M, Anderesson J, *et al*. 1999. Regulation of CCR5 and CXCR4 expression by type 1 and type 2 cytokines: CCR5 is downregulated by IL-10 in CD4-positive lymphocytes. *Clinical Immunology***. 91 (3):**254-262.

Patterson BK, Landay A, Anderesson J, *et al*., 1998. Repertoire of chemokine receptor expression in the female genital tract: Implications for human immunodeficiency transmission. *American Journal of Pathology***.153:**481-490.

Picker LJ, Singh MK, Zdraveski Z, *et al*. 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood.* **86:**1408-1419.

Pilgrim AK, Pantaleo G, Cohen OJ, *et al.* 1997. Neutralising antibody responses to human immunodeficiency virus type-1 in primary infection and long-term-non-progressive infection. *The Journal of Infectious Diseases***. 176 (4):**924-932.

Pinto LA, Sullivan J, Berzofsky JA, *et al*. 1995. ENV-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. *Journal of Clinical Investigation***. 96:** 867-876.

Poli G, Kinter A, Justement JS, *et al*. 1990. Tumor necrosis factor alpha functions in an autocrine manner in the induction of human immunodeficiency virus expression. *Proceedings of the National. Academy of Science USA.* **87:**782-785.

Poggensee G, Feldmeir H. 2001. Female genital schistosomiasis: facts and hypotheses. *Acta Tropica.* **79(3):** 193-210.

Porichis F, Vlata Z, Hatzidakis G, *et al*., 2007. HIV-1 gp120/V3-derived epitopes promote activation-induced cell death to superantigen–stimulated $CD4^+$/$CD45RO^+$ T cells. *Immunology Letters.* **108:**97-102.

Prowse SJ, Mitchel GF, Ey PL, Jenkin CR. 1978. *Nematospiroides dubius:* susceptibility to infection and the development of resistance in hypothymic (nude) BALB/c mice. *Australian Journal of Experimental Biology and Medical Science.* **56**: 561.

Quintell RJ. 2003. Genetics of susceptibility to human helminth infection. *International Journal of Parasitology.***33:**1219-1231.

Rand TH, Cruikshank WW, Center DM, Weller PF. 1991. D4-mediated stimulation of human eosinophils: lymphocyte chemoattractant factor and other CD4 binding ligands elicit eosinophil migration. *The Journal of Experimantal Medicine***. 173(6):**1521-1528.

Ranger AM, Das MP, Kuchroo VK, Glimcher LH. 1996. B7–2 (CD86) is essential for the development of IL-4-producing T cells. *International Immunology.* **(8): 10.** 1549-1560.

Rappocciolo G, Piazza P, Fuller CL, *et al.* 2006. DC-SIGN on B lymphocytes is required for transmission of HIV-1 to T- lymphocytes. Electronic*: PLoS Pathology* **2(7):** e70 doi:10.1371/journal.ppat.0020070 **.** Accessed 23-08-2008.

Reiche EMV, Nunes SOV, Morimoto HK. 2004 Stress, depression, the immune system and cancer. *The Lancet Oncology*. **5** (10): 617 – 625.

Ribeiro RM, Mohri H, Ho DD, Perelson AS. 2002*. In vivo* dynamics of T cell activation, proliferation and death in HIV-1 infection. : Why are $CD4^+$ but not $CD8^+$ T cells depleted? *Proceedings of the National Academy of Sciences of the USA.* **99 (24):**15572-15577.

Rihet P, Demeure CE,Bourgois A. 1991. Evidence for an association between human resistance to *Schistosoma mansoni* and high anti-larval IgE levels*. European Journal of Immunology***.21:** 2679-2686.

Rizzardi GP, Harari A, Capiluppi B, *et al.* 2002. Treatment of primary HIV-1 infection with cyclosporin A coupled with highly active antiretroviral therapy. *The Journal of Clinical Investigaion.* **109 (5):**681-8.

Rizzardini G, Trabattoni D, Saresella M, *et al*. 1998. Immune activation in HIV-infected African individuals. *AIDS.***12 (18):** 2387-2396.

Rodriguez-Sosa M, Satoskar AR, Calderon R, *et al.* 2002. Chronic helminth infection induces alternatively activated macrophages expressing high levels of CCR5 with low Interleukin-12 production and Th_2 biasing ability. *Infection and Immunity.* **70 (7):** 3656-3664.

Rogers PR, Croft M. 2000. CD28, OX-40, LFA-1, and CD4 modulation of Th_1/Th_2 differentiation is directly dependent on the dose of antigen. *The Journal of Immunology.***164:** 2955-2963.

Roitt IM, Brostoff J, Male DK. 1990. Immunity to protozoa and worms. In: Immunology. 2nd ed. London. Gower Medical Publishing. 17.1-17.17.

Rothenberg MA. 1998. Eosinophilia.*The New England Journal of Medicine.* **(338):**1592-1600.

Rowland-Jones SL, Dong T, Fowke KR, *et al*. 1998. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *Journal of Clinical Investigation.* **102 (9):**1758-1765.

Rulifson IC, Sperling AL, Fields PE, *et al*. 1997. CD28 costimulation promotes the production of Th_2 cytokines. *Journal of Immunology.* **158:** 658.

Saathof E, Olsen A, Sharp B, *et al*., 2005. Ecologic covariates of hookhelminth infection and reinfection in rural KwaZulu-Natal/ South Africa: a geographic information system-based study. *American Journal of Tropical Medicine and Hygiene.* **72 (4):**384-391.

Sabbaj S, Mulligan MJ, Hsieh R-H, *et al*. 2000. Cytokine profiles in seronegative volunteers immunized with a recombinant canarypox and gp120 prime-boost HIV-1 vaccine**.** *AIDS.* **14(10):** 1365- 1374.

Sabin EA, Araujo MI, Carvalho EM, Pearce EJ. 1996. Impairment of tetanus toxoid-specific Th_1-like immune responses in humans infected with *Schistosoma mansoni***.** *The Journal of Infectious Diseases.* **173 (1):**269-272.

Schottelius AJG, Mayo MW, Sartor RB, Baldwin AS. 1999. Interluekin-10 signaling blocks inhibitor of kβ kinase activity and nuclear kβDNA binding. *Journal of Biological Chemistry.* **27(4**): 31868-31874.

Schnittman, SM, Lane HC, Higgins SE, *et al.* 1986. Direct polyclonal activation of human B lymphocytes by the acquired immune deficiency syndrome virus. *Science.* **233:**1084-1086.

Secor WE, Shah A, Mwinzi PMN, *et al.* 2003. Increased density of human immunodeficiency virus type 1 coreceptors CCR5 and CXCR4 on the surfaces of $CD4^+$ T cells and monocytes of patients with *Schistosoma mansoni* infection. *Infection and Immunity.* **71 (11):** 6668-6671.

Shapira-Nahor O, Kalinkovinch A, Weisman Z, *et al.* 1998. Increased susceptibility to HIV-1 infection of peripheral blood mononuclear cells from chronically immune-activated individuals. *AIDS*. **12:** 1731-1733.

Skowron G, Cole BF, Zheng D, *et al.* 1997. gp120-directed antibody–dependent cellular cytotoxicity as a major determinant of the rate of decline in CD4 percentage in HIV-1 disease. *AIDS.***11:**1807-1814.

Skrabal K, Saragosti S, Labernardiere JL, *et al.* 2005. Human Immunodeficiency Virus Type 1 variants isolated from single plasma samples display a wide spectrum of neutralization sensitivity. *Journal of Virology.* **79:**11848-11857.

Sousa AE, Carneiro J, Meier-Schellersheim M, *et al*. 2002. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *Journal of Immunology.* **169:**3400-3406**.**

Sozzani S, Ghezzi S, Iannolo G, *et al*. 1998. Interleukin-10 increases CCR5 expression and HIV infection in human monocytes. *Journal of Experimantal Medicine.* **187 (3):** 439-444.

Spina CA, Prince HE, Richman DD.1997. Preferential replication of HIV-1 in the CD45+ RO+ memory cell subset of primary CD4 lymphocytes *in vitro*. *Journal of Clinical Investigation.* **99:**1774-1785.

Stadecker MJ, Asahi H, Finger E, Hernandez HJ, Rutitzky LI, Sun J. 2004. The immunobiology of Th1 polarization in high-pathology schistosomiasis. *Immunological Reviews*. **201(1):**168-179.

Steel C, Nutman TB. 2003. CTLA-4 in filarial infections: implications for a role in diminished T cell reactivity. *Journal of Immunology.* **170:**1930-1938.

Stevenson M, Meier AM, Mann N, *et al*. 1988. Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4^{+}cells: mechanism for persistence in AIDS. *Cell.***53:** 483-496**.**

Streblow DN, Kitabwalla M, Malkovsky M, Pauza CD. 1998. Cyclophilin a modulates processing of human immunodeficiency virus type 1 p55Gag: mechanism for antiviral effects of cyclosporin A**.** *Virology.* **245 (2):**197-202.

Tanaka M, Hirabayashi Y, Gatanaga H, *et al*. 1999. Reduction in Interleukin-2-Producing Cells but not Th1 to Th2 Shift in Moderate and Advanced Stages of Human Immunodeficiency Virus Type-1-Infection: Direct Analysis of Intracellular Cytokine Concentrations in CD4^{+} CD8^{-} T Cells. *Scandinavian Journal of Immunology.* **50 (5):** 550-554.

Tough DF, Borrow P, Sprent J. 1996. Induction of bystander T cell proliferation by viruses and type 1 interferon *in vivo*. *Science.***272:**1947-1950.

Tremblay M, Wainberg MA. 1990. Neutralisation of multiple HIV-1 isolates from a single participant by autologous sequential sera. *Journal of Infectious Diseases.* **162:** 735-737.

Turner JD, Faulkner H, Kamgno J, *et al.* 2003. Th2 cytokines are associated with reduced helminth burdens in a human intestinal helminth infection. *Journal of Infectious Diseases.* **188 (11):**1768-1175.

UNAIDS/WHO 2006 AIDS Epidemic updates. Worldwide HIV & AIDS Statistics. www.avert.org Accessed 24 July 2007.

UNAIDS/WHO Epidemiological Fact Sheets on HIV and AIDS. 2008 Updates.

Urban JF Jr, Katona IM, Finkelman FD. 1991.*Heligmosomoidespolygyrus*: CD4$^+$ but not CD8$^+$T cells regulate the IgE response and protective immunity in mice. *Experimental Parasitology.* **37(4):** 500-511.

Usui T, Preiss JC, Kanno Y, *et al.* 2006. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *The Journal of Experimental Medicine***.** **203 (3):** 755-766.

Valdez H, Carison NL, Post AB. *et al*. 2002. HIV long term non progressors maintain brisk CD8 T cell responses to other viral antigens. *AIDS***.** **16:**1113-1118.

Valdez H, Mitsuyasu R, Landay A *et al.* 2003. Interleukin-2 increases CD4$^+$lymphocyte numbers but does not enhance responses to immunization: results of A5046s. *Journal of Infectious Diseases.* **187 (2):**320-325.

Valentin A, LU W, Rosati M, *et al.* 1998. Dual effect of interleukin-4 on HIV expression: implications for viral phenotypic switch and disease progression. *Proceeedings of the National Academy of Science USA.* **95:**8886-8889.

Valentin A, Rosati M, Patenude DJ. 2002. Persistent HIV-1 infection of natural killer cells in patients receiving highly active antiretroviral therapy. *Proceedings of the National Academy of Sciences of the USA.* **(99) 10:** 7015-7020.

Viau M, Veas F, Zouali M. 2007. Direct impact of inactivated HIV-1 virions on B lymphocyte subsets. *Molecular Immunology.* **440:** 2124-2134**.**

Vos JG, Ruitenberg EJ, Van Basten N *et al.* 1983. The athymic nude rat. Immunocytochemical study to detect T-cells, and immunological and histopathological reactions against *Trichinella spiralis*. *Parasite Immunology***. 5(2):**195-215.

Wahl SM, Greenwell - Wild T, Peng G, Hale - Donze H, Orenstein JM. 1999. Co-infection with opportunistic pathogens promotes Human Immunodeficiency Virus Type 1 infection in macrophages. *The Journal of Infectious Diseases.* **179**:S457–S460.

Waldrop SL, Pitcher CJ, Peterson DM, *et al.* 1997. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency.
The Journal of Clinical Investigation. **99:**1739-1750.

Walson JL, John-Stewart G. 2007. Treatment of Helminth co-infection in individuals with HIV-1: A sysytematic review of literature. *PLoS Neglected Tropical Diseases.* **(1):3** e102.doi:10.1371/journal.pntd.0000102. Accessed on 22-04-2008

Weissman D, Poli G, Fauci AS. 1995. IL-10 synergises with multiple cytokines in enhancing HIV production in cells of monocytic lineage**.** *Journal of Acquired Immunodefieciency Syndrome and Human Retorviruses.* **9:**224-229.

Wolday D, Mayaan S, Mariam ZG, *et al.* 2002. Treatment of intestinal helminths is associated with decreased HIV plasma viral load. *Journal of Acqiured Immune Deficiency Syndromes.* **31:**56-62.

Wolmarans CT, Bremond P, de Kock KN. 2005. The occurrence and distribution of schistosome intermediate hosts in relation to the prevalence of schistosome infections in humans in a highly endemic area in the Limpopo Province, South Africa. *South African Journal of Epidemiology & Infection,* **20:**18-22.

Wynn TA, Cheever AW. 1995. Cytokine regulation of granuloma formation in schistosomiasis. *Current Opinion in Immunology.* **(7):**505-511.

Yang Y, Tikhonov I, Ruckwardt TJ, *et al.*, 2003. Monocytes treated with human immunodeficiency virus Tat kill uninfected CD4 ($^{+}$) cells by a tumour necrosis factor related apoptosis-induced ligand mediated mechanism. *Journal of Virology.* **77:**6700-6708.

Yates A, Stark J, Klein N, *et al.* 2007. Understanding the Slow Depletion of Memory CD4^{+} T Cells in HIV Infection. *PloS Medicine.***4: (5)** e177

Yazdanbakhsh M, van den Biggelaar A, Maizels RM. 2001. Th2 responses without atopy: immunoregulation in chronic helminthinfections and reduced allergic disease.*Trends in Immunology.* **22 (7)** 372-377.

Zhang M, Li X, Pang X, *et al.*, 2001.Identification of a potential HIV-induced source of bystander-mediated apoptosis in Tcells: upregulation of trail in primary human macrophages by HIV-1 tat. *Journal of Biomedical Science.* **8:** 290-296.

Zhang YJ, Fracasso C, Fiore C, *et al.* 1997. Augmented serum neutralizing activity against primary human immunodeficiency virus type1 isolates in two groups of HIV-1 infected long term non progressors **.** *The Journal of Infectious Diseases.* **176 (5):**1180-1187.

Chapter 2

Study Design, Specimen Collection and Preliminary Tests

The overall aim of the present work was to investigate immunological interactions between the HIV and helminth infections through analysis of cells obtained from singly and dually infected individuals. Collaboration with supporting laboratories and fieldwork was undertaken to obtain baseline information prior to embarking on the immunology work which constitutes the essence of the thesis.

The current chapter thus describes the study area, the participants' profile and preliminary tests that were done to categorise the participants including helminth infection versus non-infection status, serum IgE levels and HIV status. The section outlines the tests that were done by other support laboratories therefore it does not include detailed descriptive techniques. Full details of methodologies that were undertaken by the author are given in subsequent chapters.

2.1 Study's ethical clearance

This work was part of the project titled: Prospective, placebo-controlled trial of deworming of HIV infected individuals. Project: N04/02/045.The study's ethical clearance was obtained from the South African Medical Research Council's and the University of Stellenbosch's Ethics Committees in 2002 and 2004 respectively. A waiver of University of KwaZulu-Natal ethics approval was granted by the chair of the Biomedical Research Ethics Committee, Prof Wassenaar (Appendices 9, 10 and 11). Permission to conduct the study was granted by the Matthew Goniwe Clinic's management team, consisting of the director of City Health, City of Cape Town (at the time the late Dr Ivan Toms), Dr De Villiers, directly in charge the Matthew Goniwe clinic as well the sister in-charge, Sister X. Mandindi.

During the study, the core foundations of research ethics pertaining to human participants were adhered to, namely respect for dignity, right to justice, autonomy and beneficence.

2.2 Study setting and population

The study was undertaken in an informal settlement, Khayelitsha in the Western Cape Province (South Africa). Although over 90% of the participants were originally from the Eastern Cape Province, the majority had resided in Khayelitsha for a substantial number of years, with a mean stay of 10 and 14 years in the HIV positive and negative groups respectively (Adams *et al.*, 2006). Study participants were recruited between May 2002 and November 2003 for the main clinical trial till the final follow up visit and the present study analyses commenced in 2004 to 2005.

In 2004 the Khayelitsha population was estimated at 500 000 with 80% of the residents living in shacks, with a population density ranging between 2 250 and 33 786 per km^2 (mean 15 653 per km^2). In 2006, more than 14 000 households did not have access to clean water and there was almost a 30 000 sanitation backlog and an average of 105 people per toilet in sites B and C Khayelitsha (Thom, 2006). A survey of 12 primary schools in this area showed that more than 90.0% of the school children were infected by helminths (The Khayelitsha Task Team 2001), while a pilot study of helminthiasis in adults who live around Matthew Goniwe clinic (Khayelitsha) suggested levels exceeding 40% (unpublished) and a recall study on the history of helminth infection among the same group showed that more than 70.0% had been infested by helminths previously (Adams *et al.*, 2006). Within the Western Cape Province, the prevalence of HIV in Khayelitsha (22.0%) is higher than the 9,1% provincial level (Shaikh *et al.*, 2006).

2.3 Study design

This was a cross sectional study aimed at examining immunological interactions between intestinal helminth and HIV infections. Data for this study was collected at baseline of the deworming trial. Ideally, a randomly selected sample of a substantial magnitude to allow statistical power for making inferences to the entire population should have been made. However there were budgetary and some technical constraints in the face of the costs of some of the molecular tests which prevented such an approach. The study was thus designed as a pilot study for hypothesis testing within the available financial resources.

As a result, sample size for statistical power could not be taken into account while selection bias could not be optimally minimised. This is then an exploratory study and no population inferences can be made. Essentially, one of the expected outcomes is to test the hypothesis for immunological interactions between HIV and helminths and provide data that could guide the design of future similar studies.

The sampling strategy determined by the primary deworming trial. The study target population was HIV-1 positive individuals and HIV negative controls who live in an area of high helminth endemicity and limited infrastructural resources in Khayelitsha. Some sampling bias could have been introduced for the HIV positive individuals who were recruited from an HIV support group, while the HIV negative were mainly community members who were accompanying clinic attendees. The inclusion criteria for the main study group (HIV positive individuals) were the following:

- HIV sero-positivity
- Age above 18 years
- attending the HIV AIDS support group in the local Matthew Goniwe clinic
- consenting to a repeat HIV test.

The exclusion criteria were determined for both the main trial as well as for the immunology work of this thesis as some of the exclusion criteria have significant influence on the immune system activation and hence would confound the present study. The first two criteria were for the respect of patient autonomy and prevention of harm.

:

- Unwillingness to undergo an HIV test or retesting
- Known sensitivity to mebendazole
- Presence of known systemic diseases that cause immune suppression (except HIV) such as diabetes mellitus, cancer chemotherapy, prior organ transplatation, congestive heart failure, advanced chronic obstructive pulmonary disease, known liver disease or alcoholism, use of corticosteroids in excess of 10mg per day
- Known presence of other infectious diseases such as tuberculosis and sexually-transmitted infections
- Use of multivitamin supplements and prophylactic medication including immunomodulators

- Pregnancy in the last 6 months (some of the participants were on the Infant Feeding Choice programme that was in progress at the time)
- Recreational use of illicit drugs and excessive consumption of alcohol

After recruitment efforts were made to ensure optimum participation. All participants were anti-retroviral therapy-naïve at the time of study commencement as such treatment was not available then in public health institutions. The HIV-1 negative controls were recruited from the same clinic, most of whom were accompanying patients to the clinic. They were recruited for comparison purposes only, since the objective of the study was to analyse immune responses to HIV in the presence and absence of helminth co-infection. To maintain group similarity, only Khayelitsha residents were enrolled to participate.

After obtaining informed consent, 170 HIV-1 sero-positive and 65 HIV-1 sero-negative adult Khayelitsha residents were recruited into the study. Case record files and questionnaire screening were used to exclude participants who had other infectious diseases including those who were treated for active tuberculosis, sexually-transmitted infections, those who were on Bactrim prophylaxis as well as those who were taking vitamin supplements.

Thus of the 170 HIV-1 seropositive participants enrolled, 151 were eligible for analysis and donated approximately 30ml blood and/or stool samples while 45 of the 65 HIV-1 seronegatives recruited submitted these specimens. All these participants were screened for helminth infection and their peripheral blood lymphocyte profiles were phenotyped. After stratification according to HIV and helminth serology and faecal egg positivity, only samples from those participants with complete datasets were investigated further. The stratification resulted in four subgroups based on presence or absence of faecal eggs with or without elevated *Ascaris*-specifif IgE. For further immunological testing, only a limited number of samples could be analysed owing to the costs of some of the analytical tests and the budgetary limitations. From the 124 HIV^+ participants with complete data, sequential sampling was undertaken by selecting every second participant (using study identification numbers) until there were 10 participants in each of the 4 subgroups. Twenty-two HIV-negative individuals had complete data sets and were all included in subsequent analysis. Stool analysis was aimed at the identification of eggs from either *Ascaris lumbricoides* and/or *Trichuris trichuria*. As pointed out in

Chapter 1 there is significant cross-reactivity between antibodies against Ascaris and Trichuris and as there is no commercial *Trichuris trichuria* assay available the Ascaris-specific IgE test was used as proxy for infection by any one of these two pathogens. Stratification resulted in the following groups:

(i) faecal egg positivity and high *Ascaris*–specific IgE ($egg^{+}IgE^{hi}$)

(ii) faecal egg positivity and low *Ascaris* IgE ($egg^{+}IgE^{lo}$)

(iii) no eggs and elevated *Ascaris*–specific IgE($egg^{-}IgE^{hi}$)

(iv) no eggs and low *Ascaris*–specific IgE($egg^{-}IgE^{lo}$).

The last subgroup is presumably free of current and recent helminth infection while the other three subgroups have evidence of exposure to these infections. Use of serum IgE aimed at increasing the Figure 2.1 outlines the numbers of participants that were included or excluded for each selection criteria. The limitations of this selection strategy and the inherent bias in this sample are recognized.

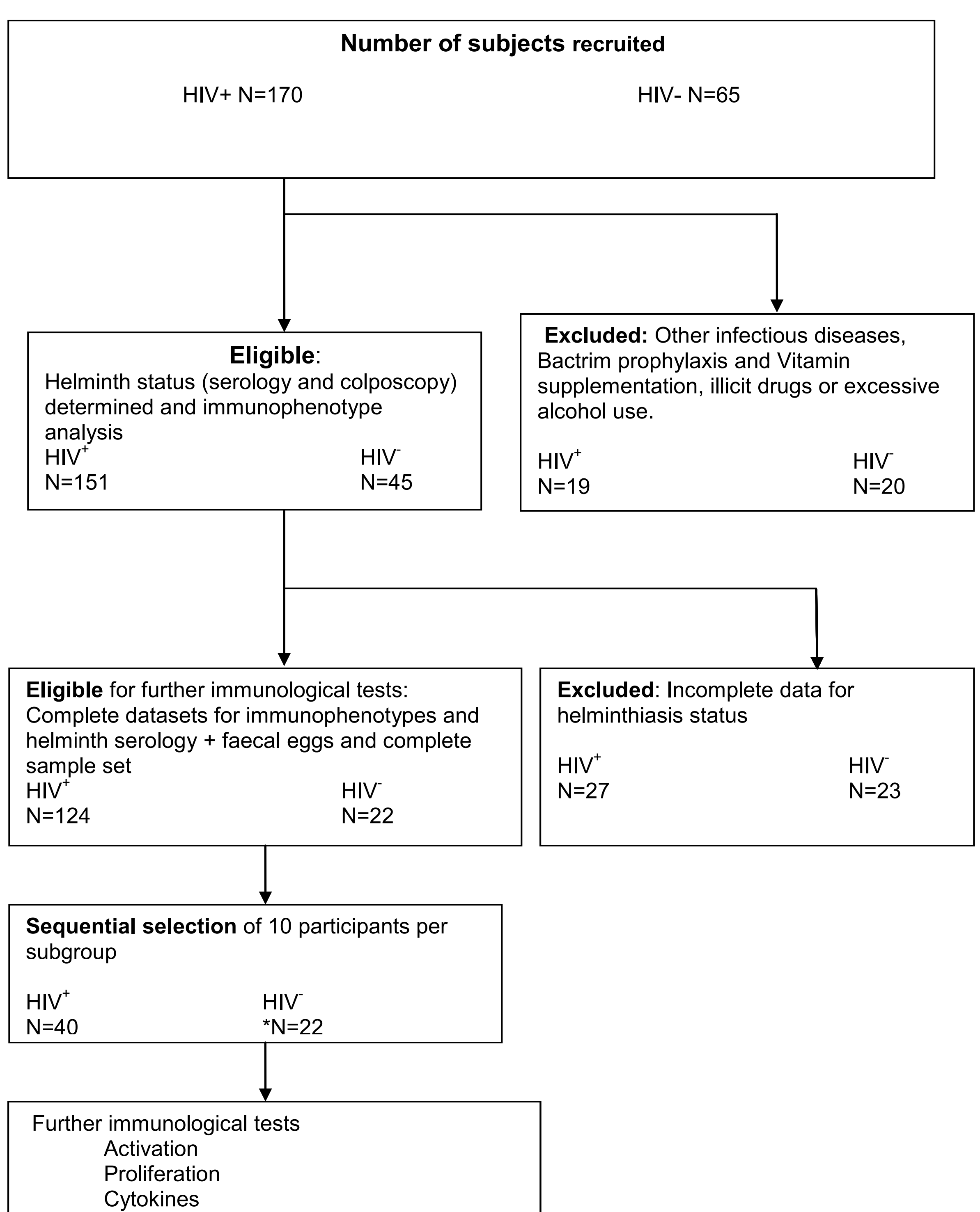


Figure 2. 1. Selection strategy for inclusion of participants for immunological analyses.
The numbers and exclusion criteria for HIV positive and negative subjects that were initially recruited into the study is illustrated. Of those consenting to participate, a proportion was excluded because they had other infectious diseases, were on Bactrim prophylaxis or vitamin supplements. Those eligible donated blood and stool samples. Helminth status was determined by both colproscopy and serology and lymphocyte immunophenotypes done. Those with incomplete data for helminth status classification were further excluded. A subset of the eligible participants was sequentially selected (every second individual) using study identification numbers for further immunological analysis. *Among the HIV^- eligible participants, 23 did not submit the complete set of samples thus the remaining 22 were included by default.

It should be noted that although efforts were made to include all the recruited HIV negative controls (n=45) for comparisons, the numbers of participants suitable for stratification of helminth infection were lower as six of the 45 paricipants did not submit stool samples. Although the samples for lymphocyte phenotypic analyses (n=38) were adequate, further immunological tests could only be conducted on a smaller subset (n=22) due to insufficient cell mumbers or poor cell viablity of less than 90%. Stratification into subgroups was therefore not possible in the HIV negative groups as one subgroup only had three participants. Thus in Chapter 3 analyses, thirty eight subjects were included while in Chapters 4-5 only twenty-two suitable participants remained. However, as the essence of this work is to assess the effect of helminth infections on immune responses to HIV, the HIV uninfected participants become less relevant in this context.

2.4 Questionnaire administration and case record file data collection

Data on health status (history of other infectious diseases), vitamin supplementation and prophylaxis treatment, demography and social habits were collected. The history of helminth infection as a child or as an adult was recorded upon interviewing the study participants. The first date of HIV testing was documented from the case record files.

2.5 Specimen collection

Blood and faecal samples were collected from each consenting individual. During each collection period, faecal samples were collected on two consecutive days to detect the presence of parasite eggs and ova. After HIV counselling was offered, blood samples were collected by standard phlebotomy and the following blood tubes were collected for the tests indicated:

- Ethylenediaminetetraacetic acid (EDTA) tube for full blood counts and lymphocyte phenotypes (5ml).
- Cell Preparation Tubes (CPT) for peripheral blood mononuclear cell (PBMC's) isolation for cryopreservation and subsequent culture experiments, determination of activation markers, proliferation and cytokines production (2 x 8ml).
- Serum Separator Tubes (SST) tubes for confirmation of HIV status, total and *Ascaris lumbricoides*-specific IgE (2x 5ml).

- EDTA tubes for plasma viral loads (5ml).

The specimens were sent to the laboratories within two hours of collection. Processing of all the samples was according to good laboratory practice and recommended laboratory protocols.

2.6 Preliminary tests

2.6 1. Detection of faecal helminth eggs and/or ova

Microscopy was performed on two stool samples collected on 2 consecutive days by two independent microscopists using formol-ether concentration and the Kato Katz methods respectively.

2.6 2. HIV tests and viral loads

Although the HIV positive participants had been tested previously before enrolling in the HIV Support group, for the purposes of this study their HIV infection status was confirmed. A rapid test for HIV (*InstantScreen*® Rapid HIV-1/2 Assay GAIFAR GmbH, Germany) was done at the clinic. Serum was re-tested at Tygerberg Academic Hospital, Department of Virology using the Abbott *Axsym* ® Microparticle Enzyme Immunoassay (MEIA) for the detection of antibodies to HIV-1 (subtypes M and O) and/or HIV-2. Confirmation of MEIA positive tests was done by PCR sequencing of viral DNA. HIV-1 viral load was determined by the Abbot LCx® HIV RNA Quantitative assay (Abbott [Q1]Laboratories, IL) in the same Virology department.

2.6.3 Full blood counts and IgE tests

Total and *Ascaris lumbricoides* specific IgE levels were determined at Pathcare Laboratories using CAP total IgE and ImmunoCAP® Specific IgE (RAST), respectively. Full blood counts (H2 Technicon) and differential counts were done by the Tygerberg Hospital Haematology Department.

2.7 Results

2.7.1 General characteristics of the participants

Of the 170 HIV-1 seropositive participants enrolled into the study, 151 donated either blood and/or faecal samples while 45 of the 65 HIV-1 seronegatives recruited submitted these specimens. Among these 196 successfully recruited participants 28 were males (their mean age was 41 years) and 170 were females (with a mean age of 32 years). The participants were classified into 2 groups according to their HIV-1 status. The general characteristics of participants are shown in Table 2.1. Fewer HIV-1 seronegative participants were recruited as clinic attendees were reluctant to undergo HIV testing as antiretroviral therapy was not available at public health institutions at the time.The HIV positive participants were already aware of their HIV status and recruitment was largely successful amongst the attendees of a HIV support group.

The HIV positive participants could not be categorised according to duration of HIV infection. Although the date of diagnosis of HIV status was retrieved from the clinic's case record files, this was found unreliable for accurate estimation of the date of seroconversion.

Table 2.1 Summary of baseline characteristics of study participants by HIV status who submitted blood and faecal samples.
The prevalence of helminth infection was estimated by microscopical screening of stools for eggs/ova as well as the levels of serum *Ascaris lumbricoides* specific IgE. Data includes sex, median ages and total serum IgE levels within the two groups.

Characteristic	HIV-1 positive	HIV-1 negative
Sex, number of participants		
Males	n =15	n =11
Females	n =136	n=34
Total	**n=151**	**n =45**
Median Age (years)	30.6	40.0
Interquertile range	25,7-35.5	30.0-48.5
Helminth egg positive (number/total) (%)]	51/124 [a] (41, 1%)	18/39 [b] (46, 1%)
Helminth egg positive and/or positive serology* (%)	99/151 (66,0 %)	32/45 (71,1 %)
Mean total IgE (Ref. range below 70KU/L)	429.2KU/L	655.18KU/L
Standard deviation	286.1	457.2
Mean Ascaris-specific IgE (Ref. range below 0.35 kU/L)	2.15kU/L	2.86kU/L
Mean Ascaris Worm Burden (SD)	170 (86.5)epg**	191 (78,8)epg
Mean Trichuris Worm Burden (SD)	19.8 (36.7)epg	74.11 (56.2)epg

*Evidence of helminth exposure as indicated by faecal egg excretion and/or elevated *Ascaris lumbricoides* specific IgE. Ascaris specific IgE cross-reacts significantly with *Trichris trichura*.

[a] Twenty seven of 151 (HIV^+) and [b] six of 45 (HIV^-) participants did not submit faecal samples. Mean worm burdens were done as egg counts per gram of stool in each person and the mean was calculated for each HIV group among the infested persons. SD= standard deviation.epg=eggs per gram of stool.

2.7.2 Prevalence of helminth infections in the Khayelitsha adult study individuals.

In both HIV groups the prevalence of helminths exceeded 40.0% when assessed by the presence of faecal eggs only and was 66.0% and 71.1% for HIV-1 positive and negative respectively, when evidence of exposure to parasites is defined by both egg excretion and elevated *Ascaris lumbricoides*-specific IgE. Some participants excreted intestinal helminth eggs with or without elevated *Ascaris lumbricoides* IgE while others had high serum *Ascaris lumbricoides* IgE but did not excrete helminth eggs in their faeces. The most common intestinal helminths in this adult population were *Ascaris lumbricoides (*44 of the 51 HIV^+egg positive and 14 of 18 HIV- egg positive participants), followed by *Trichuris trichiura (*30 of 51 HIV^+ and 8 of 18 HIV- participants). Other parasites included the *Taenia* species (5 of 51 and 3 of 18 $HIV^+$and HIV^- individuals respectively), *Fasciola* (one in each of the two HIV-1 groups) and *Schistosoma mansoni (*one of the 18 HIV-1 negatives). Among the HIV-1^+, 21 of 51 (41%) were dually infected by *Ascaris lumbricoides* and *Trichuris* and 5 of the 18 (28%) HIV- negative group harboured both these parasites. The mean Ascaris worm burdens were not significantly different in the two HIV groups while mean Trichuris burden was significantly lower in the HIV positive group (p=0.04).

Treatment with Mebendazole (and Praziquantel where necessary) was given to all participants with faecal helminth eggs. Since the faecal egg results were available earlier than IgE results which were done by another laboratory (Pathcare), Mebendazole was given to those diagnosed with fecal eggs only during the trial. However at the end of the study all participants were treated, owing to the high prevalence of helminths and unsanitary living conditions in Khayelitsha.

2.7.3 Other infectious diseases

Some of the participants had other additional infections. Of the 170 HIV seropositive individuals recruited, nineteen (11.0%), and six of 65 (9.0%) HIV seronegative participants initially recruited had tuberculosis (TB) at the time of study commencement. One HIV negative (2.0%) and 11 (7.0%) HIV $^+$ reported having sexually transmitted infections (STI) during the recruitment period.

2.7.4 Full blood counts, serum Total IgE and *Ascaris lumbricoides* specific IgE

Total IgE levels were 6-fold above the method reference range among the HIV-1 sero positive people and 9-fold higher in the HIV-1 uninfected group (Table 2.1).
Ascaris lumbricoides-specific IgE levels were considered low if the levels were below the method detection limit of 0.175 (class 0). These are based on European reference ranges for the methods. The results of *Ascaris lumbricoides* IgE are used in the following chapters together with faecal egg results to classify the individuals' helminth infection status.

The full blood count results were used for the calculation of absolute lymphocyte subset numbers in the dual-platform analysis (flow cytometry percentages and lymphocyte counts).

2.8 Discussion

Data on the prevalence of helminth infections among adults is infrequent in South Africa as most of the available survey reports are obtained from studies done on children. The present study confirms for the first time, evidence of exposure to and infection with intestinal parasites in the adult Khayelitsha residents by both faecal egg excretion and elevated *Ascaris lumbricoides*-specific IgE data. *Ascaris*-specific IgE was used as a reliable additional, serological marker of helminth exposure because there is extensive cross-reactivity between antigenic epitopes of helminths infecting humans (Bhattacharyya *et al.*, 2001) and as it is known to share antigens with *Trichuris trichiura* (Lilly-white *et al.*, 1991). At the time of conducting the study there was no crude antigen for the *Trichuris trichiura* serology. High levels of *Ascaris*-specific anti IgE would thus provide a representative serodiagnostic marker that reflects exposure to other related intestinal parasites in the population. Parasite specific IgE response was documented to appear within seven days of infection and unbound serum IgE has a short half life of two to five days while the bound antibody persists for several weeks to months in healthy nourished individuals. (Lynch *et al.*, 1998;Nagaraj *et al.*, 2004). Ten days following treatment, the levels were documented to remain high in malnourished adult males (Nagaraj *et al.*, 2004). Both *Ascaris lumbricoides* and *Trichuris trichiura* were shown to be the most prevalent helminths in this population (Adams *et al*, 2006).

The present study established that the level of exposure to helminths ranged between 66,0% and 71,1% when measured by both faecal eggs and elevated *Ascaris lumbricoides*-specific IgE among this adult Xhosa population residing in a resource-poor area. Helminth egg excretion revealed a prevalence of 41.1% and 46.1 % for HIV-1 positive and negative individuals respectively in this setting. This discrepancy highlights the point that use of egg excretion alone as an indicator of helminth infection can be misleading, particularly for studies whose aim is to analyse co-infection by helminths and other infectious agents. The mean worm burdens were slightly higher in the HIV negative groups, and Trichuris burden was significantly higher in the HIV negative group (p=0.04). This finding could corroborate the suggestion that egg excretion may be impaired by a compromised immune system.

Upon analysis of data on stool egg positivity and serum *Ascaris*-specific IgE, four groups emerged, assuming that the sensitivity of faecal egg detection was uniformly improved by use of two methods and two independent microscopists on faecal samples collected on two consecutive days. Some participants had intestinal helminth faecal eggs and high serum *Ascaris lumbricoides* IgE. This first group reflected typical, fecund helminth infection and will be subsequently referred to as $egg^{+}IgE^{hi}$.

A second group comprised of individuals who excreted helminth eggs in stool but *Ascaris* IgE levels were lower than or equalled the method detection limit (egg^{+}/IgE^{lo}). There would be two possible explanations for this combination of results: the first would be newly-acquired infection before detectable levels of antibodies are reached at the time of sample collection. Secondly, this group could represent a predominantly Th_1 phenotype, who inherently secrete lower levels of IgE and therefore are more susceptible to intestinal parasite infection (hence egg excretion). This type of distinction was more clearly defined in mice, where animals with a Th_1 phenotype were more susceptible to *Trichuris* reinfection compared to their Th_2 counterparts (Else *et al.*, 1994). Whether this phenomenon occurs in humans is yet to be elucidated. Maizels and Yazdanbakhsk (2003) described three types of phenotype outcomes that follow exposure to some helminth parasites. One of these are helminth-infected individuals who secrete lower anti-parasite IgE and higher IgG_4 levels, and have strong regulatory cell activity accompanied by high IL-10. These individuals are referred to as modified Th_2 phenotype and are susceptible to persistent, asymptomatic, usually heavy infection by parasites

and they constitute the reservoir for parasite transmission. In the present work, the egg^+/IgE^{lo} participants in the second group befit this phenotype although no data is available on IgG_4 levels for these participants.

The third study group comprised of individuals with high serum Ascaris specific IgE but absent faecal eggs (egg^-/IgE^{hi}). This group could be representing individuals with residual antibodies who had recently been exposed to the parasites and had cleared the infection; or alternatively people with a predominantly Th_2 immune phenotype with an increased potential to clear the helminth infection. According to the model presented by Maizels and Yazdanbakhsk (2003) this group represent a balanced $Th_1/Th_2/Th_3$ phenotype. The individuals in this group are apparently resistant to infections and these authors suggest that the balanced Th_1/Th_2 immune system is sufficient to eliminate invading parasites.

The last group had neither faecal eggs nor high *Ascaris* specific IgE (egg^-/IgE^{lo}). Again this group could reflect a naturally resistant subgroup (considering their living conditions that are conducive to high levels of exposure to helminth infection) or they could have been uninfected at the time of sample collection. Another possibility exists that these individuals are severely immunocompromised and fail to mount humoral (IgE) responses. Egg production by parasites (worm fecundity) is suggested to be dependent on a competent immune system (Karanja *et al.*, 1997; Viney, 2002); therefore in the proposed scenario egg and antibody production would be impaired resulting in the egg^-/IgE^{lo} status. The worm burden was generally lower in the HIV negative group compared to the HIV positive individuals, and this difference was significant for *Trichuris trichiuria.* This finding could corroborate the suggestion that egg excretion may be impaired by a compromised immune system.

Parallel to this evidence for a high level of exposure to helminth infections are the elevated serum total IgE levels (Table 2.1) in this population. Notably, although still high, the median total IgE level was lower in the HIV positive compared to the HIV negative group, probably reflecting a function of immunodeficiency. The observed hyper-IgE globulinaemia in this population concurs with the suggestion that Africans represent a genetically high IgE responder phenotype (Haus *et al.*, 1988). These workers reported highest median cord blood IgE values from African (Xhosa) neonates compared to their

White and Coloured counterparts, irrespective of maternal ascariasis or atopic family history. It is suggested that the high IgE level among the Africans is environmentally as well as genetically driven (Orren *et al.*, 1975; Gerrard, 1985). This finding could be of significance to the study hypothesis as high IgE is associated with Th_2 bias.

Finally, the immunological assays discussed in the subsequent chapters thus depended on the availability of all the relevant sample results and also on the costs of tests. For proper classification of helminth infection or non-infection, data on both egg and serology status was required in order to interpret immunological data. In addition, results of full blood counts as well as of flow cytometry were required for determination of absolute $CD4^+$and $CD8^+$ counts using the two-platform analysis. Results of immunophenotypes in Chapter 3 were only obtained from participants with complete full blood count, immunophenotyping, *Ascaris*-specific IgE and faecal microscopy results, hence the numbers declined. Further expensive assays on cryopreserved PBMCs (Chapters 4 and 5) were done on 40 HIV^+ and 22 HIV^- participants where suitable samples were available (however cytokine analysis was only done on the 40 HIV^+ group because of costs).

References.

Adams VJ, Markus MB, Kwitshana ZL, *et al.* 2006. Recall of intestinal helminthiasis by HIV-infected South Africans and avoidance of possible misinterpretation of egg excretion in helminth/HIV co-infection analyses. *BMC Infectious Diseases.* **6 (88):** 1-8.

Anonymous. 2001. The Khayelitsha Task Team: *Building health partnerships that work*. ISBN1-919809-26-0. Brochure published by the Khayelitsha Task Team. Obtainable from the Medical Research Council, Cape Town, South Africa.

Bhattacharyya T, Santra A, Debendra N. et al ., 2001. Possible approach for serodiagnosis of Ascariasis by evaluation of Immunoglobulin G4 response using *Ascaris lumbricoides* somatic antigen. *Journal of Clinical Microbiology.* **39 (8):** 2991-2994.

Else K J, Finkelman FD, Maliszewski CR, Grencis RK. 1994. Cytokine-mediated regulation of chronic intestinal helminth infection. *Journal of Experimental. Medicine.* **179:**347-351.

Gerrard JW. 1985. Genetic factors in the development of asthma. *In* E. B. Weiss, M. S. Segal, and M. Stein, editors. *Bronchial Asthma- Mechanisms and Therapeutics,* 2nd ed. Little Brown, Boston. 24-29.

Haus M, Heese H De V, Weinberg EG, Potter PC, Hall MJ, Malherbe D. 1988. The influence of ethnicity, an atopic family history and maternal ascariasis on cord blood serum IgE concentrations. *J Allergy Clin Immunol.* **82:**179-189.

Karanja DMS, Colley DG, Nahlen BL, *et al.* 1997. Studies on Schistosomiasis in Western Kenya : I. Evidence for immune-facilitated excretion of schistosome eggs from patients with *Schistosoma mansoni* and Human Immunodeficiency Virus co-infections. *American Journal of Tropical Medicine and Hygiene.* **56 (5) :**515-521.

Lilly-white JE, Bundy DAP, Didier JM, *et al.* 1991. Humoral immune responses in human infection with the whipworm *Trichuris trichiura*. *Parasite Immunology.* **13:**491-507.

Lynch NR, Hagel IA, Palenque ME, Di Prisco MC, Escudero JE, Corao LA, Sandia JA, Ferreira LJ, Botto C, Perez M, Le Souef PN. 1998. Relationship between helminthic infection and IgE response in atopic and nonatopic children in a tropical environment. *Journal of Allergy and Clinical Immunology* **101**:217-221.

Maizels RM, Yazdanbakhsh M. 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature Reviews Immunology.* **3:**733-744.

Nagaraj S, Raghavan R, Macaden R, Kurpad AV. Intestinal parasitic infection and total serum IgE in asymptomatic adult males in an urban slum and efficacy of antiparasitic therapy. *Indian Journal of Medical Microbiology.* **22** (1):54-56

Orren A R, Walls S, Dowdle EB. 1975. Serum immunoglobulin E concentrations of allergic patients and blood donors: influence of allergy, sex and race on IgE values. *South African Medical Journal.* **49:** 1387-1390.

Shaikh N, Abdullah F, Lombard CJ, Smit L, Bradshaw D, Makubalo L. 2006. Masking through averages-intraprovincial heterogeneity in HIV prevalence within the Western Cape. *South African Medical Journal.* **96: (6)** 538-543.

Thom A. 2006. Widening gulf between Khayelitsha and Cape Town. www.health-e.org.za/news/article.php?uid=20031372 Accessed 8 Sept. 2009.

Utzinger J, Botero-Kleiven S, Castelli F, Chiodini P. L. *et al.* 2009. Microscopic diagnosis of sodium acetate-acetic acid-formalin-fixed stool samples for helminths and intestinal protozoa: a comparison among European reference laboratories. *Clinical Microbiology and Infection.***16** (3):267-273.

Viney M. 2002. Developing worms need the host immune system. *Trends in Parasitology.* **18 (2):** 57.

Chapter 3

Ex-vivo peripheral blood lymphocyte phenotyping, eosinophil counts and their relationship to viral load

3.1 Introduction

The present chapter analyses and compares cellular phenotypes (and viral load) among subgroups of HIV-1 positive and negative individuals with or without helminth co-infection. A brief overview of outcomes of both infections on cellular proportions and phenotypes is re-contextualised below.

3.1.1 Lymphocyte subsets and viral load in HIV infection

3.1.1.1 $CD4^+$ lymphocytes:

As described in Chapter 1, HIV infection is classically characterized by marked reduction and eventual depletion of $CD4^+$ and a gradual rise in $CD8^+$ lymphocytes with disease progression while total $CD3^+$ counts remain constant (Giorgi, 1993). On this basis, guidelines for initiation and monitoring of HIV therapy are founded on $CD4^+$ lymphocyte thresholds (Center for Diseases Control (CDC), 1997). Lymphocyte immuno-phenotyping thus plays a pivotal role in defining the rate of cellular immune dysfunction.

3.1.1.2 $CD8^+$ cells:

Cytotoxic or suppressor T lymphocytes ($CD8^+$) play a key role in the control of viral infections. Likewise a significant increase of these cells occurs within the first few months of HIV infection and remains elevated during the asymptomatic phase (Koup *et al*., 1994). At the onset of AIDS, absolute numbers of total, $CD4^+$, $CD8^+$ lymphocytes decrease, but the percentage of $CD8^+$ increases (Giorgi, 1993). At the end stage of the disease all T lymphocytes are $CD8^+$ (Giorgi, 1993). Different ratios of viral load to $CD8^+$ lymphocyte numbers have been reported (Jin *et al*., 1999; Metzner *et al.,* 2000; Betts *et al*., 2001) thus the association between $CD8^+$ frequencies and disease progression remains controversial.

3.1.1.3 NK Cells:

Changes in NK cell numbers occur within the functional phenotypes, as alluded to in Chapter 1. The number of $CD56^{+}CD16^{+}$ NK cells (the most potent cytolytic phenotype) decreases dramatically. The expression of CD16 also drops with a subsequent expansion of CD16 dim $CD56^{-}$ cells. This results in impaired NK cell functional activity in AIDS disease (Hu *et al*., 1995; De Souza *et al.*, 2000; Kotttilil *et al*., 2004; Mavillo *et al*., 2005; Meier *et al*., 2005). With regard to NK cell numbers, literature reports discrepant results during HIV infection. It is therefore not clear whether their numbers increase or decrease with disease progression.

3.1.1.4 B cells:

B cell numbers can be increased by polyclonal stimulation by HIV proteins. The significance of enumeration of this lymphocyte pool during HIV infection has not been well established (Kunkl, 1997).

3.1.1.5 Viral Load:

Molecular level data indicates that from primary infection through the asymptomatic phase to disease onset, there is continuous change in the mean viral activity (Bagnarelli *et al.*1994). On average, viral load quantification enables a relative measure of virus replication and cell killing (Wei *et al*., 1995). Consequently, despite the wide individual (intra-host) variability, viral loads are strongly associated with and widely used to assess disease progression (Nolte, 1999). It is noteworthy however that findings of a recent report suggest that viral load is not always an accurate predictor of $CD4^{+}$ cell decline (and disease progression) at the individual level (Nelson, 2006).

While there are several studies suggesting that ethnic variation in viral load occurs, mainly indicating that Black Africans generally have lower levels compared to other ethnic groups (Rizzardini *et al*., 1998, Anastos *et al*., 2000; Saul *et al.*, 2001, Swindells *et al*., 2002; Smith *et al*., 2003), some workers however have not observed these race differences. In a US study assessing racial differences in viral load among White, Black and Hispanic military members, Brown and colleagues (1997) did not observe race

dependent variations in their study participants. It is not known whether the antiretroviral treatment contributed to this finding because the participants were receiving antiretroviral treatment. Nonetheless, the ethnicity and virus threshold level issues should probably be considered in interpreting viral load studies and still warrant further verification.

3.1.2 Helminths and peripheral blood cellular distributions:

The hallmark of cellular response to helminth infection is eosinophilia and mastocytosis. In addition, however disruptions to T cell populations similar to those occurring in HIV infection (decreased $CD4^+$ and increased $CD8^+$ lymphocytes) have been demonstrated in animal experimental helminthiasis (Else and Grencis, 1991, 1996; Soltys *et al.*,1999) and in humans: Pollack and colleagues (1993) described low $CD4^+$ counts in HIV seronegative Ethiopian Jews who had immigrated to Israel from Ethiopia. The high prevalence of helminth infection in this group was suggested to be associated with the $CD4^+$ lymphocytopaenia. Many studies were carried out particularly in this immigrant population of Ethiopians to Israel. Kalinkovich *et al*. (1998) ascribed decreased $CD4^+$ and increased $CD8^+$ lymphocyte counts in helminth infected HIV uninfected Ethiopian groups. These authors confirmed this association when eradication of helminths resulted in reversal of the immune alteration.

With the suggested role of helminth-induced immune dysregulation in African populations, other studies followed. A study in Tanzania determining the reference values of lymphocyte subsets in HIV seronegative adult Tanzanians reported low percentages of $CD3^+$ and $CD4^+$ and $CD4^+$ $CD8^+$ ratios compared to the levels reported for European adults (Urassa *et al.,* 2003). Similarly, Smith and colleagues (2003) reported lower $CD4^+$ counts among Black Africans compared to Caucasians in an observational study of 537 antiretroviral naïve HIV-1 positive individuals attending two East London (Britain) clinics. All these studies speculatively suggested that a general tendency towards low $CD4^+$ mostly accompanied by elevated $CD8^+$ lymphocytes is found among African populations, owing to chronic exposure to infectious agents, predominated by helminths. However, some immunological studies undertaken in other African studies have not found these alterations in T cell subsets: These include Nigeria (Aina *et al.*, 2005), Zimbabwe (Katzenstein *et al.*, 1990), Senegal (Le Guenno *et al.*, 1991), Cote d' Ivore (Kestens *et al.*, 1992) and the Gambia (Whittle *et al.*, 1993).

Rizzardini *et al.*, (1998) compared lymphocyte profiles, among others, of HIV infected Ugandans and Italians with those of HIV uninfected Ugandan and Italian controls and found similar percentages of $CD3^{+}CD8^{+}$ and $CD3^{+}CD4^{+}$ and $CD4^{+}$ $CD8^{+}$ ratios between the controls and HIV infected groups.

Contrary to the studies citing a trend towards low levels of $CD4^{+}$ counts among Africans, in West Africa, higher levels were reported among 380 HIV infected adults compared to 370 French participants. Approximate equivalencies of absolute $CD4^{+}$ counts based on adjusted percent values were about a third higher in the West Africans than in French adults (Anglaret *et al.*, 1997). Furthermore, healthy Ugandan adults (Tugume *et al.*, 1995; Lugada *et al.*, 2004) and uninfected Tanzanian individuals (Levin *et al.*, 1996) had mean $CD4^{+}$ counts that are markedly higher than those found in healthy Ethiopians.

These studies confirm evidence of significant geographic and ethnic variations that are postulated to play a role in the different haematologic and immunologic parameters, further endorsing a recommendation that local standardization of reference values and threshold decision levels should be undertaken (Anglaret *et al.*, 1997).

3.1.3 Flow Cytometry

"Flow cytometry is a unique, powerful and technologically sophisticated methodology that utilizes principles of physics, biology, chemistry, engineering, digital electronics, optics, analog devices and laser technology to measure the light scattering and fluorescent characteristics of particles" (Walker, 1998). As a result of these features, flow cytometry has wide applications in various fields.

In immunology, flow cytometry combines the specificity and stable binding of monoclonal antibodies with the unique properties of laser and flourescent light to quantify, characterise/identify target molecules and monitor function in cells.

Basically, laser light illuminates single particles (within cells) in liquid suspension (in a fluidic system). As they pass through the light path, they scatter the laser beam. The size and texture of the particles will determine the amount of light scattered to the sides (side scatter (SSC) and forwards (forward scatter (FSC). The laser will simulteneously excite flourescence in antibodies conjugated to fluorochromes, the intensity of fluorescence

being directly proportional to the amount of target molecule bound by the antibodies. The specific fluorescent light from fluorochrome-labelled monoclonal antibodies and scattered laser light is collected in different channels for each stained particle/cell. The FSC, SSC and flourescent light is collected via a series of lenses, specific filters and beam splitters and directed to photomultiplier tubes (photodiode for FSC). These photodetector tubes then relay the signals and convert the light to electrical signals which are finally digitalized by the Analog to Digital Converter (ADC), placed into specific channels corresponding to the level of fluorescence and stored in the computer system. This allows for graphic analysis and interpretation of stored data after acquisition. The data may be displayed in a linear or logarithmic scale depending on its distribution (Davey and Kell, 1996). Earlier, flow cytometers used a single laser for the light source (Neon, Argon), and the technology advanced to the use of dual lasers in most instruments (BD Biosciences, 2004).

Flow cytometry allows qualitative and quantitative analysis of multi-fluorochrome-labeled molecules on cell surfaces as well as intracellular molecules such as cytokines, hormones, DNA, proliferation antigens as well as functional aspects such as membrane potential determination (Walker, 1998). These can be determined at single cell level. Simultaneous multicolour staining and use of dual laser technology allows up to five parameter analysis within a single cell, thus results in generation of enormous amounts of quantitative and qualitative data from small quantities of sample. These unique features make flow cytometry highly reliable, cost effective, specific, dynamic and reproducible.

To obtain optimum benefits from the numerous advantages of flow cytometry, the investigator needs to gain adept insight to use the sophisticated technique. This includes appropriate experiment planning and design, proper instrument settings including accurate compensation for spectral overlap and attention to detail during acquisition.

In the present work, flow cytometry was used to differentiate lymphocyte populations (immunophenotypes), to assess the level of expression of activation markers on cell surfaces and functional (proliferation) aspects in lymphocytes.

3.1.4 Objectives of the chapter

In view of the fact that helminths have been documented by others to be associated with a similar pattern of low $CD4^+$ and increased $CD8^+$ counts as in HIV infection, the assumption is that dual infection by both infectious agents would have an additive aberrant effect on the lymphocyte population distribution and proportions. In line with the study hypothesis, disease progression (as determined by viral load and absolute $CD4^+$ counts in this section) would be enhanced by co-infection with helminths and HIV. The current section aimed to determine whether infection by helminths could be associated with alterations in peripheral blood lymphocyte subsets and eosinophils (and subsequently impair effective responses to HIV infection). Total lymphocytes and their subpopulations (T, B, NK, $CD4^+$ and $CD8^+$), eosinophil counts and viral load among groups of HIV infected and uninfected participants with or without helminth co infection were described in a South African setting. The secondary objectives were to elucidate whether if present, these immune alterations affected the HIV disease progression in terms of correlations between viral load, $CD4^+$ and $CD8^+$ counts in singly- and dually-infected individuals.

3.2 Methods

3.2.1 Participants and samples

All participants with other infectious diseases (TB and STI's) were excluded for this and all subsequent analyses. Clinical examinations by the study clinician, questionnaire responses and case-record files were used to screen for these infections. At the time of study the clinic was using an empirical (syndromic) approach to management and treatment of STIs, hence the prescribed treatments as appearing on patient files was also used as additional screening information (provided by the collaborating clinic clinician) for the presence of STIs. Similarly TB treatment data was used as additional screening information. Thus immunophenotype data from EDTA anticoagulated whole blood of 149 HIV-1 positive participants and 45 HIV-1 negative controls were analysed in combination with the full blood counts that were done at the Tygerberg Hospital Haematology department.

3.2.1.1 Methodology description

This description is adapted from the Becton & Dickenson Biociences (BD) Product Catalog Techical Resources (2003)[a]. The MultiTest four- colour, two-tubes panel method employing direct immunofluorescence to determine the percentages of T lymphocyte ($CD3^+$) subsets ($CD3^+CD4^+$) and ($CD3^+CD8^+$) as well as non-T lymphocytes ($CD16^+/56^+$ NK cells and $CD16^+$ B cells) was used. The two panels of monoclonal antibodies (mAbs) to human lymphocyte surface antigens already labeled with fluorochromes as listed in Table 3.1 were purchased from BD. Panel 1 MultiTEST™ includes monoclonal antibodies to CD3/CD4/CD8/CD45 and panel 2 MultiTEST™ monoclonal antibodies to CD3/CD19/ CD16 and CD56/ CD45.

Table 3.1 List of monoclonal antibodies and fluorochromes.
The monoclonal antibodies to lymphocyte surface antigens (their clones in brackets) and the fluorochromes with which they are labelled are tabulated. The abbreviations of the fluorochromes are given and will be used in all subsequent text. The respective channels on the flow cytometer (FL1-FL4) where each fluorochrome is measured are shown in brackets.

Cell Surface Marker(clone)	Fluorochromes	Full Name
CD3 (SK7)	FITC (FL1)	Flourescein Isothiocyanate
CD4 (SK3)	APC (FL4)	Allophycocyanin
CD8 (SK1)	PE (FL2)	Phyco-erythrin
$CD16^+$56B (73,1 & NCAM16,2)	PE (FL2)	Phyco-erythrin
CD19(SJ25C1)	APC (FL4)	Allophycocyanin
CD45 (2D1-HLe-1)	PerCP (FL3)	Peridinin chlorophyll protein

3.2.1.2 Optimisation

The BD/ Pharmingen staining procedure for labeling cell surface markers on whole blood was optimized to determine if microtiter plates could be substituted for the tubes without compromising the efficacy of the assay. Five samples were stained in tubes, v-bottom and u-bottom microtiter plates. The results of all the 5 samples were highly reproducible.

An illustration of one of the samples is depicted in Table 3.2. Thereafter all subsequent staining procedures were done on v-bottom tubes.

Table 3.2 Reproducible results obtained from staining in different containers.
Typical reproducible results obtained from the same sample that was stained using different methods. All tests were performed in duplicate. Cells were stained with a cocktail of fluorochrome-conjugared monoclonal antibodies to CD45, CD3, CD4, CD8, CD 16/56 and CD19. The sample was stained in tubes, V-bottom and U-bottom microtiter plates. The values are expressed as percentages of positive cells as determined by flow cytometry. The table represents data from one sample.

Lymphocytes (%)	Tubes	U-bottom	V-bottom
CD3	85	85	85
CD4	25	25	25
CD8	54	52	54
NK	1	2	2
B	5	6	4
T h/s ratio	0.45	0.46	0.45
Lymphosum	96.3	95.7	96.5
CD3 difference*	1	2	0

NK: natural killer lymphocytes; T h/s: T helper/suppressor ratio; B lymphocytes. *CD3 lymphocyte difference.

3.2.1.3 Procedure for Staining

All reagents were equilibrated to room temperature. (NB: Cold lysing solution would not lyse the cells adequately). Excess glove powder was removed by rinsing gloved hands in tap water to avoid particulate interference with flow cytometry analysis. Monoclonal antibodies were diluted 1:3 in sterile Phosphate Buffered Saline (PBS) pH 7.2 (Sigma) prior to each experiment. Each specimen was assigned to appropriate wells of a V bottomed 96-well microtiter plate (Costar Corning) for each of the antibody panels 1 and 2. Twenty microliters of Panel 1 mAbs and 20μL of Panel 2 mAbs were added to appropriately designated wells of the microtiter plate, ensuring that the mAbs solutions were added to the bottom of the well. The EDTA blood was mixed thoroughly by gently inverting the tubes five times. Fifty microliters of the well-mixed blood was pipetted into the designated wells containing antibody solutions. The antibody solutions were thoroughly mixed with blood by using a pipette. The plate was covered and incubated in the dark at room temperature for 15 minutes. After incubation, the plate was centrifuged

at 4°C at 300 ±30g for 3 minutes. The excess supernatant was carefully aspirated by low pressure vacuum and discarded. Using the multi-dispensing pipettor, 180 µl of 1x FACS lysing solution (BD) diluted in sterile distilled water, was added to all wells and mixed by gently shaking the plate in the IKA MTS Multiwell plate shaker (Sigma) at 1000rpm for 30 seconds. The plate was covered and re-incubated in the dark at room temperature for 10 minutes after which it was centrifuged for 3 minutes at 4°C at 300 ±30g to pellet the cells. Diluted haemoglobin was removed from each well by aspiration as above. The cell pellets were resuspended by addition of 180µL of filtered PBS to each well and by gentle mixing. The plate was covered and centrifuged for 3 minutes at 4°C at 300 ±30g. The supernatants were aspirated and discarded. The wash/centrifugation steps were repeated one more time. The stained cells were then resuspended in 180µl of fixative (4% v/v formaldehyde (Sigma) in filtered PBS). The samples were transferred to disposable, polystyrene 12x75mm BD FACS tubes containing 450µl of 4% v/v formaldehyde in filtered PBS. These could be kept at 4ºC in the dark for up to 24 hours or analysed immediately on the FACS Calibur™ (BD) Neon-Argon dual laser flow cytometer, using the MultiSET software program. Where necessary, the Cell Quest software was used to reanalyze discordant results.

3.2.1.4 Flow cytometry analysis

The flow cytometer was calibrated using the Calibrite™ APC Beads Target Card (BD) and the 3 colour Calibrite™ beads (BD) using the FACSComp™ software to set the Photomultiplier tube (PMT) voltage gain, detectors amplification and spectral overlap compensation. The threshold was adjusted to minimise debris just before running the samples. Acquisition of samples then followed and for each sample the two sets of panels of antibodies were analysed in two tubes that were vortexed beforehand.

The MultiSET ™ software then determined the percentages of lymphocytes in each sample: Firstly a leucocyte gate was established on the side scatter versus $CD45^+$ PerCP region wherein lymphocytes were identified as CD45 bright cells with low side scatter. From this region all T lymphocytes were gated on the basis of CD3 expression ($CD3^+$). T helper/ inducer ($CD3^+$ $CD4^+$) and T suppressor/ cytotoxic ($CD3^+$ $CD8^{+)}$ were then determined from these gates (Figure 3.1). Similar gating was established for the

second tube. From the leucocyte gate in the side scatter versus $CD45^{+}$ PerCP and side scatter versus CD3 plots, all CD3 negative lymphocytes, NK cells ($CD3^{-}$ $CD16^{+}CD56^{+)}$ and B lymphocytes ($CD3^{-}$ $CD19^{+}$) were computed. A double-platform method was then used to calculate the absolute counts of each lymphocyte subset from the total lymphocyte counts (from Haematology counts) and the lymphocyte percentages obtained by flow cytometry. Figure 3.1 illustrates the gating or selection of lymphocyte subsets.

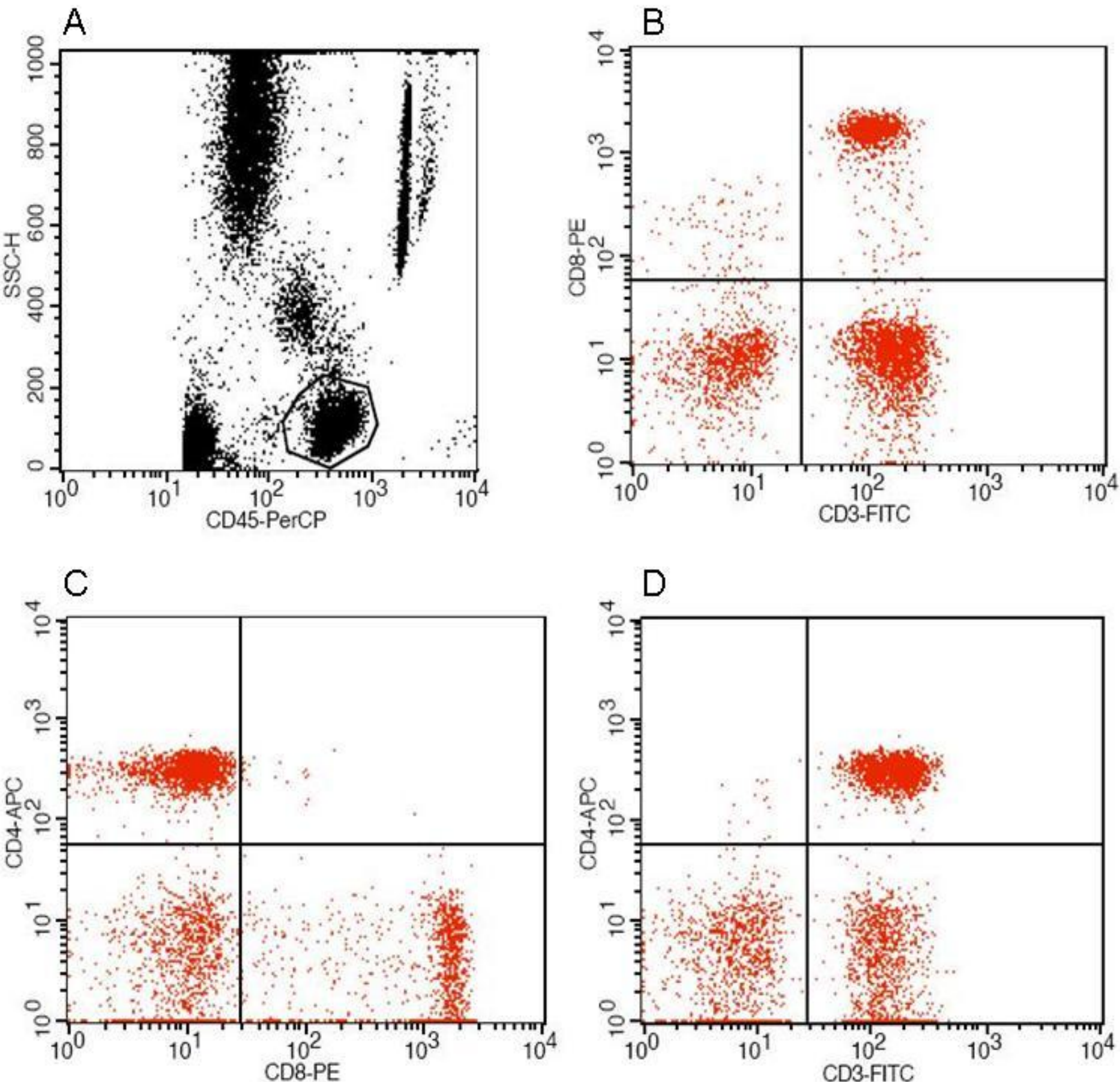


Figure 3.1 Gating strategy for identification of lymphocyte subsets.
The gating strategy for identifying $CD4^{+}$ and $CD8^{+}$ lymphocytes from the combined regions is illustrated. Whole EDTA blood was stained with fluorochrome-conjugated monoclonal antibodies to CD45, CD3, CD4, CD8, CD19, CD16 and CD56 and analysed with MultiTest software in the FacsCalibur flow cytometer. Leucocytes are gated and identified as CD45 bright cells with low side scatter (SC) on the SC versus CD45 dot plot (A) and lymphocytes then gated by CD3 staining (B & D). T helper/ inducer ($CD3^{+}$ $CD4^{+}$), panel D and T suppressor/ cytotoxic ($CD3^{+}$ $CD8^{+}$) panel B were determined (upper right quadrants) from these gates. Panel C would indicate the proportion of double positive (CD4+ CD8+) lymphocytes (upper right quadrant). Similarly NK and B lymphocytes were gated as CD3- $CD16^{+}$ plus $CD56^{+}$ and (CD3-$CD19^{+}$ respectively (not shown). The representative sample was obtained from a healthy donor.

3.2.1.5 Quality control

- To check that almost all the lymphocytes were included in the gate, the total lymphocyte sum (total T, B and NK) (lymphosum) accepted was as close as possible to 100 (the acceptable lymphosum is between 90 and 110%).
- To establish minimal tube to tube variation, the accepted percentage $CD3^+$ lymphocyte difference between the two tubes was less than 3%.

3.2.1.6 Statistical analysis

Statistica 7.0 and NCSS 2004 software were used for the statistical analyses. The median was used as a measure of central tendency for descriptive statistics. The normal distribution was tested by the Shapiro Wilks tests and variables with skewed data were log-transformed. As some of the variables remained skewed after this transformation non-parametric tests were used. Kruskal Wallis ANOVA was used to test the medians and for multiple comparisons of all measured variables between the HIV positive and the HIV negative subgroups. Findings were considered significant at the level of $p \leq 0.05$.

3. 3 Results

3.3.1 Summary characteristics of the HIV-1 positive and the HIV-1 negative participants.

The results of this section are divided into two, the first part relates to all the participants whose EDTA blood samples were analysed for both full blood counts and phenotyping of lymphocyte subsets (Table 3.3). In the second part the results are analysed for those participants with complete data for helminth status stratification as well as lymphocyte phenotypes.

Of the 151 HIV-1 positive participants who submitted blood samples, a total of 149 EDTA bloods were analysed for phenotypes and 124 had complete data for stratification, while 45 of the 65 HIV-1 negative participants submitted bloods for phenotyping and 39 were suitable for stratified analysis. The demographic, viral and immunologic profile of all the participants are summarized in Table 3.3 and highlight the important immunologic

indices such as $CD4^+$ and $CD8^+$ counts for the 2 groups in this population. Expectedly, the HIV positive group medians were mostly lower than the HIV negative values (except indeed for higher $CD8^+$ values in HIV^+ group).

In subsequent sections, median absolute numbers of lymphocyte subsets, eosinophil counts and viral load are compared within HIV-1 positive and negative subgroups stratified according to stool egg positivity and *Ascaris lumbricoides*-specific IgE (*Ascaris* IgE) as in section 3.2.2. The parameters are analysed to determine if helminth exposure results in anomalies that can be associated with impaired immune responses to HIV.

Table 3.3 Characteristics and immunological indices for HIV-1 and HIV-1 negative groups. Demographic characteristics and immunological indices for HIV-1 positive and HIV-1 negative groups are shown. EDTA blood was used for full blood counts using H2 Technikon haemocytometer and flow cytometric analysis of lymphocyte phenotypes after staining with monoclonal antibody cocktails conjugated to fluorochromes. Separate samples were used to determine viral loads. Where applicable reference values are shown in brackets and these have been established within the local community served by the Tygerberg Academic Hospital (Tygerberg Hospital Haematology Dept).

CHARACTERISTICS	**HIV-1 POSITIVE**			**HIV-1 NEGATIVE**		
Males	15			11		
Females	134			34		
Total	**149**			**45**		
	Median	**25%**	**75%**	**Median**	**25%**	**75%**
Age (years)	**29.4**	25.7	35.5	**41.2**	30.0	48.5
Eosinophils (0.2- 0.6 $x10^9$/L)	**0.14**	0.07	0.27	**0.24**	0.11	0.39
Total lymphocytes (1.0-4.0 $x10^9$/L)	**1.98**	1.41	2.48	**2.22**	1.91	2.81
T lymphocytes (1.1-1.7cells/ml)	**1.49**	1.06	1.86	**1.71**	1.33	1.97
$CD8^+$(0.5-0.9 $x10^9$/L)	**0.78**	0.56	1.01	**0.47**	0.29	0.58
$CD4^+$ (0.7-1.1 $x10^9$/L)	**0.32**	0.29	0.47	**0.74**	0.59	0.86
CD4:CD8 ratio 1-1,5	**0.38**	0.25	0.58	**1.58**	1.34	2.36
NK cells (0.2-0.4 $x10^9$/L)	**0.11**	0.06	0.19	**0.18**	0.11	0.25
B lymphocytes(0.2-0.4 $x10^9$/L)	**0.11**	0.07	0.17	**0.25**	0.21	0.35
Viral load (copies/ml)	**33317**	**170**	**120288**			

25%, 75%= 25th and 75th percentiles respectively. The reference values as established for the local population at Tygerberg Hospital in brackets. NB: The local lower limits differ from those of the CDC Reference ranges for $CD4^+$: 0,475-1,616 $x10^9$/L; and $CD8^+$:0,209- 0,924 $x10^9$/L (NHANES 2003-2004) http://www.cdc.gov/nchs/data/nhanes/nhanes0304/103cmetCD4=Cd8+

3.3.2 Characterisation of subgroups by helminth infection and HIV status

Excretion of parasite eggs by infested hosts is intermittent and stool microscopy has limited diagnostic sensitivity, examination of a single stool specimen can result in false results and misclassification of ihelminth infection (van Gooi *et al.*, 2003). Furthermore, egg excretion is also a function of a competent immune system (Karanja *et al.*, 1997) and worm burden (May and Anderson, 1979).The intensity of infection is documented to peak in children between the ages of five and fifteen years and is lower in adults (Bundy, 1990), a phenomenon associated with the development and maturation of immune responses following prolonged exposure. In addition, hosts that harbour non-fecund and male species may not excrete eggs. For these reasons we proposed that sole reliance on the presence of parasite eggs in stool to diagnose helminthiasis can be misleading, particularly in adults and result in serious misinterpretation of co-infection studies (Adams *et al.*, 2006). Furthermore, Maizels and Yazdanbakhsh (2003) presented three phenotypic outcomes of helminth infection that are determined by T helper cell and antibody isotype (IgG_4 and IgE) profiles. Each phenotype is characterised by specific immune responses to helminths. Stool egg detection was therefore supplemented with serum *Ascaris lumbricoides* –specific IgE measurement to increase diagnostic sensitivity of micrposcopy and delineate the serological phenotypes.

Based on *Ascaris*-specific IgE levels and stool helminth-egg excretion, the two main groups of participants ($HIV\text{-}1^+$ and $HIV\text{-}1^-$) were then further stratified into different categories depending on their evidence of exposure to helminth infection (egg excretion with or without elevated serum *Ascaris* -specific IgE) as follows:

- Group 1 (egg^+/IgE^{hi}): Trichuris and/or Ascaris egg positive stool and elevated *Ascaris* IgE (n =21)
- Group 2 (egg^+/IgE^{lo}): Trichuris and/or Ascaris egg positive stool without elevated *Ascaris* IgE (n=35)
- Group 3 (egg^-/IgE^{hi}): Trichuris and Ascaris egg negative stool but elevated *Ascaris* IgE (n =21)
- Group 4 (egg^-/IgE^{lo}): Trichuris and Ascaris egg negative stool and without elevated *Ascaris* IgE (n=47)

Among the $HIV\text{-}1^-$ the groups were:

Group 5 (egg^+/IgE^{hi}): n=9; Group 6 (egg^+/IgE^{lo}): n=9; Group 7 (egg^-/IgE^{hi}): n=11; Group 8 (egg^-/IgE^{lo}): (n=10).

Use of egg excretion alone as an indicator of helminth infection can be misleading, particularly in adults for several reasons. Firstly the sensitivity of stool microscopy is low and because excretion of intestinal parasite eggs is intermittent (van Gooi, 2003) studies whose aim is to analyse co-infection by helminths and other infectious agents.

3.3.3 Immunophenotype profiles of subgroups in HIV^+ and HIV^- groups

To evaluate any differences between groups with HIV-helminths co-infection and those without, the median values for the various variables were desribed within the subgroups as tabulated in Tables 3.4 and 3.5 for HIV positive and negative groups respectively.

Table 3.4 Median values for eosinophils, viral load and lymphocyte subsets in HIV-1+ subgroups.

Median, minimum and maximum values of lymphocytes, eosinophils and viral loads in HIV+ groups classified as either excreting/not excreting helminth eggs- with or without elevated *Ascaris* IgE. Data represent results of full blood counts, viral load determination and ex vivo phenotyping of whole blood obtained from these HIV+subgroups. Whole EDTA blood was stained with fluorochrome-conjugated monoclonal antibodies to CD45, CD3, CD4, CD8, CD19, CD16 &CD56 and percentages of cells expressing these surface markers determined by flow cytometry. Absolute CD4+, CD8+, numbers were obtained by multiplying the absolute counts by percentages per total counts. Cells are expressed in count x 10^9/L. and viral load in mRNA copies /ml.

	EOSIN	**TOT LY**	**T LY**	**CD8**	**CD4**	**CD4: CD8**	**NK LY**	**B LY**	**V-LOAD**
Group 1 egg$^+$/IgE^{hi} n = 21									
Median	**0.32a**	**1.72**	**1.30**	**0.66**	**0.28a**	**0.38a**	**0.11**	**0.09a**	**70878**
25%	0.1	1.56	1.1	0.58	0.15	0.28	0.09	0.06	41 449
75%	0.79	1.9	1.58	0.93	0.43	0.44	0.18	0.11	120 288
Group 2 egg$^+$/IgE^{lo} n = 35									
Median	**0.15b**	**2.1**	**1.75**	**0.84**	**0.41b**	**0.48b**	**0.12**	**0.15b***	**25666**
25%	0.07	1.61	1.21	0.58	0.27	0.37	0.06	0.09	1886
75%	0.25	2.8	2.25	1.02	0.66	0.8	0.19	0.22	70 615
Group 3 egg$^-$/IgE^{hi} n = 21									
Median	**0.18b**	**1.89**	**1.39**	**0.78**	**0.32b**	**0.37a**	**0.10**	**0.12b**	**87813**
25%	0.1	1.54	1.24	0.57	0.16	0.27	0.06	0.08	4929
75%	0.3	2.06	1.73	0.99	0.46	0.69	0.21	0.16	165 421
Group 4 egg$^-$/IgE^{lo} n =47									
Median	**0.10b**	**1.98**	**1.25**	**0.66**	**0.23a**	**0.34a**	**0.11**	**0,10b**	**28258**
25%	0.06	1.15	0.83	0.43	0.12	0.17	0.06	0.05	4178
75%	0.23	2.3	1.8	1.1	0.39	0.55	0.18	0.14	199 859
***All HIV+ n=149**									
Median	0,14	1.98	1,49	0,78	0,32	0,4	0,1	0,1	33317

Legend: 25%, 75%= 25th and 75th percentiles respectively.Eosin: eosinophils;Tot Ly Total lymphocytes; T ly T-lymphocytes; CD4:CD8 ratio. *All HIV$^+$ are the overall median values obtained from all the 45 HIV negative participants inTable 3.2; CDC reference ranges- CD4+: 0,475-1,616 x10^9 cells/L; CD8+:0,209- 0,924x10^9 cells/L (NHANES).The CD4+ and CD8+ ranges for the HIV negative group 8 without evidence of helminth exposure (presumably representing healthy controls for this population) fall below the CDC reference ranges, albeit the small number of individuals in this group (N=10). Subgroups with significant interactions shown in red while similar groups are indicated with similar letters.

Table 3.5 Median values for eosinophils and lymphocyte subsets in HIV-1^{-} subgroups. Median, minimum and maximum values of lymphocytes subsets and eosinophils in HIV^{-} groups stratified by stool helminth eggs (microscopy) with or without elevated *Ascaris* IgE.Data represent results of full blood counts determination and from ex vivo phenotyping of whole blood obtained from these HIV-subgroups. Whole EDTA blood was stained with fluorochrome-conjugated monoclonal antibodies to CD45, CD3, CD4, CD8, and CD19, CD16 and CD56 and percentages of cells expressing these surface markers determined by flow cytometry. Absolute lymphocyte numbers were derived from multiplying the percentages by total counts from full blood counts. Cells expressed in count x10^{9} /L

	Eosin	*Tot ly	*T ly	CD8	CD4	CD4: CD8	NK ly	B ly
Group 5 egg^{+}/IgE^{hi} n = 9								
Median	**0.37a**	**1.95a**	**1.40**	**0.36**	**0.67**	**1.86**	**0.08**	**0.22**
25%	0.24	1.67	1.29	0.20	0.55	1.52	0.08	0.2
75%	0.45	2.18	1.71	0.56	0.72	2.2	0.18	0.23
Group 6 egg^{+}/IgE^{lo} n=9								
Median	**0.12b**	**2.65b**	**1.91**	**0.49**	**0.75**	**1.54**	**0.17**	**0.29**
25%	0.08	2.17	1.52	0.38	0.66	1.26	0.16	0.27
75%	0.2	2.81	2.0	0.59	0.79	2.19	0.22	0.37
Group 7 egg^{-}/IgE^{hi} n =11								
Median	**0.31a**	**2.87b**	**1.73**	**0.47**	**0.68**	**1.37**	**0.23**	**0.25**
25%	0.15	2.01	1.63	0.29	0.58	0.98	0.17	0.14
75%	0.56	2.97	2.22	0.77	0.93	2.36	0.27	0.34
Group 8 egg^{-}/IgE^{lo} n =10								
Median	**0.22b**	**2. 79b**	**1.83**	**0.41**	**0.76**	**1.85**	**0.16**	**0.24**
25%	0.10	1.8	1.33	0.29	0.68	1.5	0.09	0.21
75%`	0.29	2.79	2.0	0.54	0.89	2.36	0.28	0.44
All HIV $^{-}$ n=45								
Median	0,2	2,22	1,71	0,47	0,74	1,58	0,18	0,24

Legend: 25%, 75%= 25th and 75th percentiles respectively. Eosin: eosinophils;Tot Ly Total lymphocytes; T ly T-lymphocytes; H/S helper/suppressor ratio. *All HIV^{-} are the overall median values obtained from all the 45 HIV negative participants inTable 3.2; CDC reference ranges- CD4+: 0,475-1,616 x10^{9} cells/L; CD8+:0,209- 0,924x10^{9} cells/L (NHANES).The CD4+ and CD8+ ranges for the HIV negative group 8 without evidence of helminth exposure (presumably representing healthy controls for this population) fall below the CDC reference ranges, albeit the small number of individuals in this group (N=10). Subgroups with significant interactions shown in red while similar groups are indicated with similar letters.

3.3.4 Interactions between variables in subroups

3.3.4.1. The HIV-singly-infected (egg$^-$gE^{lo}) group does not differ from the HIV and typical helminth co-infected (egg$^+$IgE^{hi}) and all dually infected subgroups.

According to the study hypothesis, the HIV-1$^+$, egg$^-$/IgE^{lo} group should have a relatively competent immune profile as the group is singly-infected by HIV while the other three subgroups have evidence of dual infection. Contrary to expectation, it is noteworthy that the egg$^-$/IgE^{lo} group had the lowest median absolute CD4$^+$ cell and lymphocyte counts. Additionally, when comparing the egg$^+$/IgE^{hi} group with the egg$^-$/IgE^{lo} group, there were no significant differences in median values for all the measured variables except for eosinophils which were significantly higher in the dually infected individuals (Table 3.4 and Figure 3.2 in page 109) In contrast, the helminth uninfected group had lower T lymphocytes, CD4$^+$ counts, CD4:CD8 ratio and similar levels of CD8$^+$ and NK lymphocyte counts when compared to the group with typical helminthiasis (egg$^+$/IgE^{hi}).

The HIV negative, helminth uninfected (egg$^-$IgE^{lo}) and the typical helminth infected (egg$^+$IgE^{hi}) subgroups:

Higher median absolute counts for all T and non-T lymphocyte subsets and similar CD4:CD8 ratios were observed when the egg$^-$gE^{lo} was compared to the typical helminth infected subgroup (egg$^+$/IgE^{hi}), Total lymphocyte counts were significantly higher ($p=0.05$) in the egg$^-$gE^{lo} group and a lower trend ($p=0.09$) in eosinophils was present.
In both HIV positive and negative groups, the egg$^-$/IgE^{lo} typically had lowest eosinophil counts as eosinophilia is mainly associated with helminth infection.

3.3.4.2. The HIV positive, egg$^+$/IgE^{hi} group shows a tendency to lymphocytopaenia and significant eosinophilia.

Amongst the subgroups with evidence of dual infection, in the HIV$^+$, helminth eggs$^+$ and high Ascaris IgE (egg$^+$/IgE^{hi}) group, the median absolute values were lower for all lymphocyte populations, compared to the overall absolute count medians for the entire HIV positive group and also lower compared to the egg$^+$/IgE^{lo} and the egg$^-$/IgE^{hi} groups

(Table 3.4 page 103). The lower values reached significance for B lymphocytes (p=0.02) between this egg^{+}/IgE^{hi} and the egg^{+}/IgE^{lo} groups (Figure. 3.2 Panel H).

The egg^{+}/IgE^{hi} status was also associated with more frequent eosinophilia compared to the other three subgroups and this difference was statistically highly significant (p=0.01) between this egg^{+}/IgE^{hi} group and the egg^{-}/IgE^{lo} group (no evidence of worm exposure) Figure. 3.2 panel A.

The median viral load for the egg^{+}/IgE^{hi} group appeared to be more than 2-fold higher than that of the entire HIV^{+} group and nearly 3-fold higher than those of the egg^{+}/IgE^{lo} and egg^{-}/IgE^{lo}subgroups (Table 3.4). These were however not statistically significant (p=0.12). It is noted that the $egg^{-}IgE^{hi}$ subgroup had the highest viral load.

The HIV negative, $Egg^{+}IgE^{hi}$ group displays lymphocytopaenia and eosinophilia.

As in the HIV positives, among the dually infected subgroups, the HIV uninfected, egg^{+}/IgE^{hi} participants had lower median values for all lymphocyte subsets except the CD4:CD8 ratio that was highest (Table 3.5). Total lymphocytes were significantly lower in the egg^{+}/IgE^{hi} compared to the egg^{-}/IgE^{hi} group (p=0.05) (Figure 3.3 in Appendix 1).

Furthermore, the egg^{+}/IgE^{hi} group had marked eosinophilia (Table 3.5) compared to the rest of the HIV-1 negative subgroups. Absolute eosinophil counts also showed a higher trend in the egg^{+}/IgE^{hi} group compared to the group without evidence of helminth infection (egg^{-}/IgE^{lo} group (p=0.09) (Figure 3.3).

3.3.4.3 Low IgE and presence of stool eggs is associated with higher $CD4^{+}$ counts, lower viral load and a tendency towards an increase in all lymphocyte populations.

The HIV+, Egg^{+} IgE^{lo} group

The egg^{+}/IgE^{lo} participants had highest median values for all measured variables (Table 3.4. Differences in $CD4^{+}$, and CD4:CD8 ratio medians were highly significant (p=0.01, and p=0.03 respectively) between the egg^{+}/IgE^{lo} and the egg^{-}/IgE^{lo} groups (Figure 3.2

panels E and F). B lymphocyte median values also differed significantly between this egg^+/IgE^{lo} and the egg^+/IgE^{hi} subgroups (p=0.02) (Table 2b).

Additionally, the median absolute numbers of all the lymphocyte subsets were even higher than the overall values for the entire HIV positive group (Table 3.4).

Finally, the egg^+/IgE^{lo} group also had the lowest median viral load (Table 3.4) although this was not statistically significant (p=0.12).

The HIV- , Egg^+ IgE^{lo} group:

In the HIV negative group, among the helminth infected subgroups, the egg^+/IgE^{lo} participants appeared to have the highest median T-,CD4+, $CD8^+$ and B lymphocyte counts. The median $CD4^+$ was similar to the values for the egg^-/IgE^{lo} group (without evidence of helminth infection) (Table 3.5 page 104). However, no statistical significance was reached for these observations.

Other observations in this section included the findings that low IgE also typically is accompanied by low eosinophil counts, particularly in the HIV uninfected groups.

3.3.4.4 High IgE was associated with higher viral loads in the HIV positive group

Among the HIV infected participants, both groups with elevated IgE (the egg^+/IgE^{hi} and egg^-/IgE^{hi} groups) had highest median viral loads (70 878 copies per ml (cpml) and 87 813 cpml respectively) compared to the two groups with low *Ascaris* IgE -egg^+/IgE^{lo} and egg^-/IgE^{lo} (25 666 cpml & 28 257 cpml respectively Table 3.4). Although these differences in viral load were not statistically significant, the two groups with high IgE had almost three-fold higher median values.

High IgE and eosinophilia were associated with lower $CD4^+$ counts in the HIV negative group

Likewise, among the HIV uninfected individuals, both groups with high IgE (egg^+/IgE^{hi} and egg^-/IgE^{hi}) had marked eosinophilia and lower median $CD4^+$ counts (Table 3.5).

In summary,:

- The egg^-/IgE^{lo} and the egg^+IgE^{hi} subgroups did not differ in the HIV infected group except for significantly lower eosinophil counts, while in the HIV negative group all measured variables except the CD4:CD8 ratio, were lower in the egg^+IgE^{hi},compared to the egg^-/IgE^{lo} subgroup.
- The egg^+/IgE^{hi} helminth infection was associated with generalised reduction of all lymphocyte populations irrespective of HIV status, amongst the helminth infected subgroups.
- High IgE was accompanied by higher viral load in the HIV positive group; while it was related to eosinophila and lower $CD4^+$ counts in HIV uninfected individuals.
- The stool egg positive group with low IgE had higher median T- and non-T lymphocytes and lowest median viral load in the HIV positive group while this was true for T lymphocytes, $CD8^+$ and B lymphocytes in the HIV negative group. Median $CD4^+$ count was similar to that of the helminth-uninfected subgroup.

With the exception of significant reduction in B cells in $HIV^+/egg^+/IgE^{hi}$ group, no marked differences were observed in NK and B cell populations in the other subgroups (Tables 3.4 and 3,5 pages 103- 104).

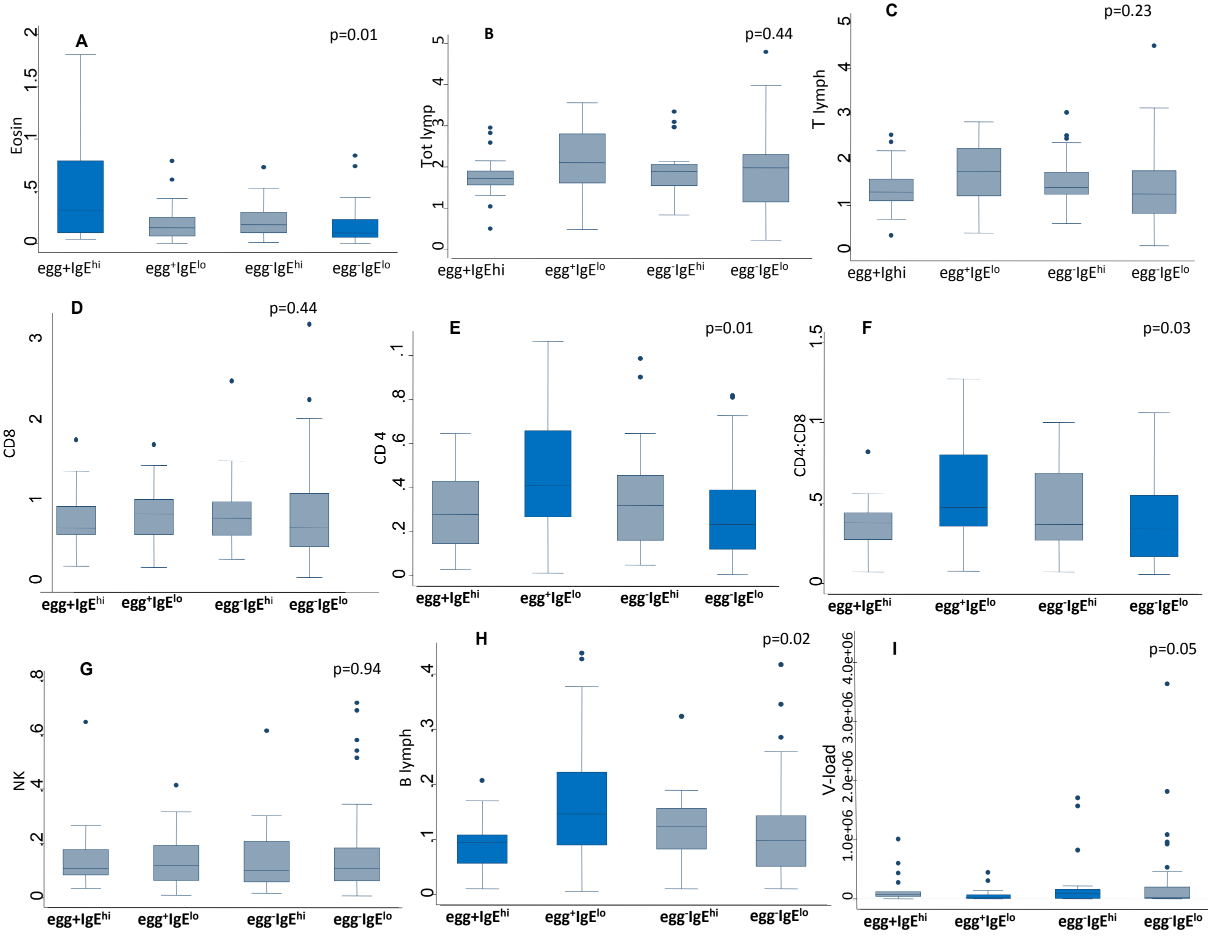


Figure 3.2 Kruskal Wallis Box and Whisker Plots for lymphocyte phenotypes in HIV+ groups.

Whole EDTA blood was stained with fluorochrome-conjugated monoclonal antibodies to CD45, CD3, CD4, CD8, CD19, CD16 and CD56 and percentages of cells expressing these surface markers determined by FacsCalibur flow cytometry using the MultiTest software. Full blood counts were done for eosinophil counts and dual platform calculation of absolute lymphocyte counts. Viral load was determined by PCR.

Egg^{+} IgE^{hi} group (n = 17) stool egg positive and elevated serum *Ascaris lumbricoides*-specific IgE; Egg^{+} IgE^{lo} group (n=35) stool helminth egg positive and low *Ascaris lumbricoides* IgE; Egg^{-} IgE^{hi} group (n=21) No stool egg excretion and high *Ascaris lumbricoides* IgE; Egg^{-} IgE^{lo} group (n=47) No stool egg excretion and low *Ascaris lumbricoides* IgE. Significantly different group /s shown in deep blue. The Y axis shows the absolute cell counts per milliliter of blood, and viral load in copies per milliliter of blood.

Table 3.6 Kruskal Wallis ANOVA, Median tests and multiple comparisons of eosinophils and lymphocytes in HIV^{+} groups.

Lymphocyte phenotypes were determined using flow cytometry. Percentage medians of each cell population were compared by Kruskal Wallis ANOVA for significant differences in each of four HIV^{+} subgroups. Two tailed comparative p values are shown for all significant interactions.

Variable	N	KruskalWallis ANOVA by rank p values	Median Test p values	Multiple Comparisons 2 tailed p values	*Groups contributing to the significant p values
Eosinophils	124	0.009	0,014	0,013	*1 & 4
Total Lymphs	124	0,506	0,619	0,512	
T-lymphocytes	124	0,240	0,909	0,167	
CD8+	124	0,661	0,178	0,561	
CD4+	124	0,012	0.026	0,011	*2 & 4
H/S ratio	124	0.030	0.032	0,035	*2 & 4
NK cells	124	0,826	0.673	0,712	
B Lymphs	124	0.030	0.112	0.095	1 & 2
Viral load	119**	0,041	0.008	0.056	*2 & 3

*Group 1 – Egg^{+}IgE^{hi} ; *Group 2- Egg^{+}IgE^{lo} *Group 3- Egg^{-}IgE^{hi} *Group 4- Egg^{-}IgE^{lo} . Egg- faecal helminth eggs. IgE^{hi} - high serum *Ascaris lumbricoides*-specific IgE; IgE^{lo} – low serum *Ascaris lumbricoides*-specific IgE NB.** If extreme values for viral load are excluded from analysis the interaction becomes significant, otherwise it is not significant when all viral load values are included.

3.3.5 Maximum Viral loads and Correlates of immunodeficiency among HIV positive participants

To determine the interaction of exposure to helminths and the parameters of advanced HIV disease in this population, the level of immunodeficiency was compared between the four subgroups of participants with and/or without HIV-helminth co-infections. The recognised indicators of immunodeficiency in HIV disease, namely level of $CD4^+$ depletion and viral loads were used to grade disease severity because there is no follow up data in this cross-sectional study to assess progression of disease. The international thresholds (CDC, 1993) of onset of immunodeficiency (<0,5 cells $x10^9$/L) and advanced/severe immunodeficiency, (<0,2 cells $x10^9$/L) were used to categorise the groups for level of disease progression. The overall group median (0,32 cells $x10^9$/L) is similar to the CDC threshold of 0,35 $x10^9$/L identified as a level below which functional immune deficiency is present in resource-poor settings (World Health Organization (WHO), 2005; AIDS Education and Training Centers (AETC), 2006), thus this cut-off level was also used to grade the immunodeficiency (as moderate) in the subgroups of the study population. For viral loads, the first level of grading was based on the overall median for the group (33 000 copies/ml) and the cutoff levels of 100 000 and $1x10^6$ copies/ml were used in Table 3.7 to further characterise the immunodeficiency.

The proportions of individuals with viral loads exceeding 100 000 in each subgroup were: 33% (Egg^+IgE^{hi}); 17% (egg^+IgE^{lo}); 48% (egg^-IgE^{hi}) and 29% (egg^-IgE^{lo}). In the latter, three individuals had virus burden exceeding 1 000 000 cpm. The egg^+IgE^{lo} group had the highest proportion of participants (19 of 35) with less than 33 000 copies/ml and none of the group individuals had virus concentrations exceeding 1000 000 copies/ml (Table 3.7).

Grading by $CD4^+$ counts showed that the the percentages of individuals with severe and moderate immunodeficiency were 62% (egg^+IgE^{hi}); 31% (egg^+IgE^{lo}); 56% (egg^-IgE^{hi}) and 70% (egg^-IgE^{lo}).The egg^+IgE^{lo} group had the lowest proportion of individuals with severe immunodeficiency (14% versus 33% and 38% in the other three groups) and the group had the highest number of individuals with $CD4^+$ counts >0.5 cells $x10^9$/L than the other three subgroups (Table 3.7).Figure 3.3a

In dual infection, low $CD4^+$ counts frequently associated with high virus burden. In all three subgroups with evidence of dual infection, almost all individuals with < 0.2 cells $x10^9$/L had viral loads exceeding 100 000 copies per millilitre. However, in the egg^-IgE^{lo} subgroup, 13 of 18 individuals with < 0.2 cells/L had viral load less than 100 000cpm. (Results not shown).

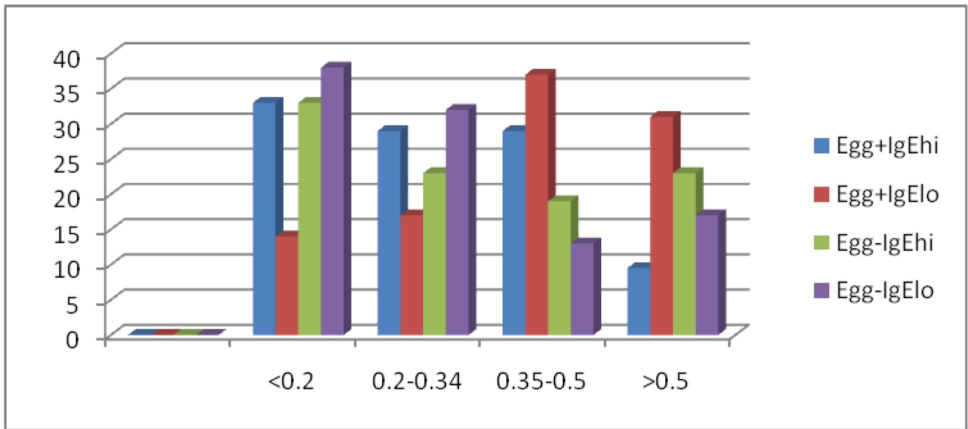


Figure 3.3a Grading immunodeficiency by CD4 + count levels. Percentages of individuals in subgroups of HIV+ with different grades of immunodeficiency measured by CD4+ counts categories fourHIV and helminth infection subgroups. The Y axis shows the percentages of individuals in each subgroup while the X axis represents the levels of absolute CD4+ counts per ml.

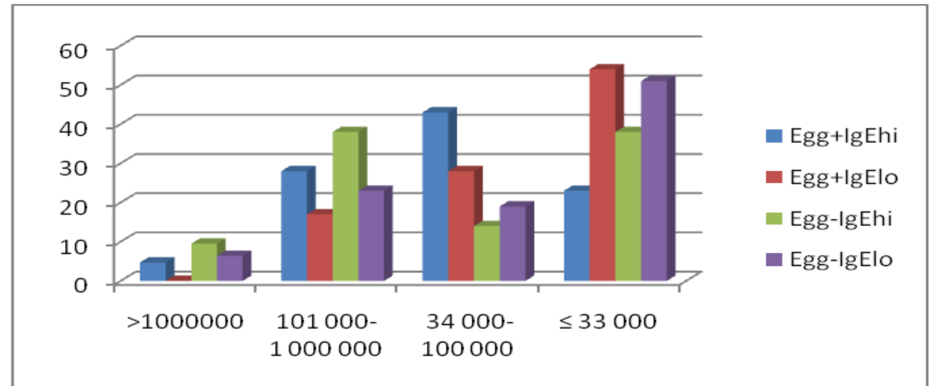


Figure 3.3b Grading of viral load levels in the four subgroups of HIVand helminth infection. .Percentages of HIV+ individuals in co-infected subgroups with different categories of virus burden. The Y axis shows the percentages of individuals in each subgroup while the X axis represents the levels of virus burden in copies per ml.

In summary, grading of immunodeficiency by $CD4^{+}$ counts and viral load did not reveal significant differences between the egg^{+}/IgE^{hi} and the $egg^{-}IgE^{lo}$ groups. However, there were relatively higher numbers of individuals with CD4+ above 0.5 cells/ml and more individual with virus burden below 33 000 cpm in the $egg^{-}IgE^{lo}$ subgroup (figures 3.3a and 3.3b) . The egg^{+}/IgE^{lo} group participants consistently had lower proportions of individuals with advanced immunodeficiency in terms of both $CD4^{+}$ counts and viral loads. None of the egg^{+}/IgE^{lo} group participants had virus concentrations equal to or greater than 10^{6} copies/ml, while the maximum load was lowest in this group and highest in the egg^{-}/IgE^{lo} group, without evidence of helminth infection (Table 3.7).

Both groups with high IgE (the egg^{+}/IgE^{hi} and egg^{-}/IgE^{hi} groups) had the higher percentages of individuals with viral load exceeding 100 000 cpml (33% and 48% versus 17% and 29% in the two low IgE groups). No differences in proportions of individuals with immunodeficiency were noted between egg^{+}/IgE^{hi} group and the group without evidence of exposure to helminths (egg^{-}/IgE^{lo}).

Table 3. 7 Proportions of HIV^{+} participants grouped by helminth infection and their HIV disease stage surrogate markers.

Whole EDTA blood was stained with monoclonal antibodies conjugated to fluorochromes and analysed by flow cytometry. Full blood count results were used to derive absolute lymphocyte counts. Viral load was determined by PCR. CD4^{+} absolute counts and viral load threshold data were then established and used to define the level of HIV disease in each of the 4 subgroups. The numbers (and percentages) of individuals within each threshold category are tabulated in each subgroup.

CD4^{+} counts (cells/uL)	**Egg^{+}IgE^{hi}** N=21	**Egg^{+}IgE^{lo}** N=35	**Egg^{-}IgE^{hi}** N=21	**Egg^{-}IgE^{lo}** N=47
<0.2	7 (33%)	5 (14%)	7 (33%)	18 (38%)
0.2-0.34	6 (29%)	6 (17%)	5 (23%)	15 (32%)
0.35-0.5	6 (29%)	13 (37%)	4 (19%)	6 (13%)
>0.5	2 (9.5%)	11 (31%)	5 (23%)	8 (17%)
Viral Load (copies/ml)				
>1 000 000	1 (4.7%)	0	2 (9.5%)	3 (6.4%)
101 000-1 000 000	6 (28%)	6 (17%)	8 (38%)	11 (23%)
34 000-100 000	9 (43%)	10 (28%)	3 (14%)	9 (19%)
≤ 33 000	5 (23%)	19 (54%)	8 (38%)	24 (51%)
Maximum Viral load	**1 013 265**	**448 447**	**1 711 249**	**3 637 244**

*Egg^{+}: presence of faecal helminth eggs. Egg^{-}: no faecal helminth eggs. IgE^{hi}: high serum *Ascaris lumbricoides*-specific IgE. IgE^{lo} : low serum *Ascaris* IgE.

Four levels of CD4 count threshold were set to grade immunodeficiency, ie <0.2, 0.2-0.34; 0.35-.5;>0.5 x10^{9} cells/L to represent Aids defining severe, moderate ,onset and immunocompetent respectively. Also, four cutoff levels of virus burden were set at ≤33 000, 34 000-100 000;101 000;1 000 000 and > 10^{6} RNA copies/ml for the overall median burden in the group, increased, therapy initiation and AIDS defining levels respectively. (AETC, 2006)

3.3.6 Correlations between Viral Load, CD4^{+} and CD8^{+}counts.

The relationship between virus burden and the number of immune cells targeted by HIV (CD4^{+} cells) and the number of CD8^{+} cells were assessed as a proxy indicator of disease progression in the four subgroups. The correlation in the subgroups

(representing the different infection phenotypes) were evaluated to determine if advancing disease (for instance a strong negative correlation between $CD4^+$ and viral load) would be associated with certain groups of co-infected individuals.

The relationships among viral load, $CD4^+$ and $CD8^+$ counts were assessed by Spearman Rank (r) correlation.

3.3.6.1 Correlation between $CD4^+$ and viral load

The correlation between CD4+ counts and viral load was determined within the entire HIV-1^+ group and within each of the 4 subgroups by the Spearman rank correlation.

Table 3.8 a Correlation between $CD4^+$absolute counts and viral load.

HIV subgroups	N	r	p-value
1(Egg^+ IgE^{hi})	21	-0.61	0.01
2 (Egg^+ IgE^{lo})	35	-0.53	0.01
3 (Egg^- IgE^{hi})	21	-0.69	0.01
4 (Egg^- IgE^{lo})	47	-0.38	0.01
All groups	124	-0.51	0.01

*Egg^+: presence of faecal helminth eggs; Egg^-: no faecal helminth eggs; IgE^{hi}: high serum *Ascaris lumbricoides*-specific IgE; IgE^{lo}: low serum *Ascaris* IgE.

Overall there was a significant negative correlation (r= -0.51; p<0.05) between viral load and $CD4^+$ counts when all the HIV^+ participants were analysed as one group. This inverse correlation was stronger in both groups with high Ascaris IgE (the egg^+/IgE^{hi} and egg^-/IgE^{hi} groups) and moderate in the egg^+/IgE^{lo} group while in the egg^-/IgE^{lo}group the negative correlation was significant but weakest (Table 3.8a)

3.3.6.2 Correlation between $CD4^+$ and $CD8^+$ absolute counts

Correlation between $CD4^+$ and $CD8^+$ was determined in all the 124 HIV-1^+ individuals and within each of the 4 groups by the Spearman rank correlation.

Table 3.8 b Correlation between $CD4^+$ and $CD8^+$absolute counts

HIV subgroups	N	r	p-value
1(Egg^+ IgE^{hi})	21	0.72	0.01
2 (Egg^+ IgE^{lo})	35	0.56	0.01
3 (Egg^- IgE^{hi})	21	0.38	0.09
4 (Egg^- IgE^{lo})	47	0.54	0.01
All groups	124	0.54	0.01

*Egg^+: presence of faecal helminth eggs; Egg^-: no faecal helminth eggs; IgE^{hi}: high serum *Ascaris lumbricoides*-specific IgE; IgE^{lo}: low serum *Ascaris* IgE.

A significant positive correlation ($r=0.54$; $p<0.05$) was found in all 124 HIV positive participants between $CD4^+$ and $CD8^+$ counts. The egg^+/IgE^{hi} group had the strongest relationship between the two variables while this was moderate and similar in both groups with low IgE. The correlation was weakest in the egg^-IgE^{hi} group 3 (Table 3.8b).

3.3.6.3 $CD8^+$ vs viral load.

The correlational relationship between $CD8^+$ and viral load was determined within the entire group $HIV\text{-}1^+$ and within each of the 4 groups by the Spearman rank correlation.

Table 3.8 c Correlation between $CD8^+$ and viral load.

HIV subgroup	N	r	p-value
1(Egg^+ IgE^{hi})	21	-0.56	0.02
2 (Egg^+ IgE^{lo})	35	-0.06	0.75
3 (Egg^- IgE^{h}i)	21	-0.10	0.67
4 (Egg^- IgE^{lo})	47	0.03	0.84
All groups	124	-0.07	0.43

*Egg^+: presence of faecal helminth eggs; Egg^-: no faecal helminth eggs; IgE^{hi}: high serum *Ascaris lumbricoides*-specific IgE; IgE^{lo}: low serum *Ascaris* IgE.

Although there was a very weak inverse correlation between $CD8^+$ cells and viral load when all the HIV positive individuals were analysed as a group, only the egg^+IgE^{hi} group showed a stronger negative correlation ($r= -0.56$; $p<0.05$) between the two variables (Table 3.8c).

3.4 Discussion

The current chapter analysed lymphocyte proportions, eosinophils and viral loads in relation to HIV-1 with or without helminth co-infections. In addition to faecal egg excretion, elevated *Ascaris lumbricoides* specific IgE was also used to strengthen the distinction between helminth infection and non-infection and stratify immune phenotypes on the basis of egg excretion and specific IgE levels. Possible associations between HIV-helminth co-infection and compromised responses to HIV were assessed in terms of differences in lymphocyte counts (and in particular $CD4^+$ lymphocytes) and viral load between groups with or without dual infections. Correlates of HIV disease progression in the various subgroups were also evaluated in terms of correlations between $CD4^+$, $CD8^+$ counts and viral load.

According to the study hypothesis, the immune profile of the HIV-singly-infected individuals without any evidence of worm infection (egg^-IgE^{lo}) would be relatively competent compared to their dually-infected counterparts. Findings in the present section showed significantly lower eosinophils in the egg^-IgE^{lo} when compared to the typical helminth infected, egg^+IgE^{hi} . No other significant differences between this egg^-IgE^{lo} and the egg^+IgE^{hi} subgroup was found, although the singly infected HIV positive subgroup had comparatively more individuals with lower virus burden and CD4+ counts above 0.5 cells/ml. In the absence of HIV infection, a tendency to higher T and non-T lymphocytes (significantly higher in total lymphocytes and a lower trend in eosinphils) was observed between the egg^-IgE^{lo} and the typical helminth infected egg^+IgE^{hi} subgroup, although the rest of the differences were not statistically significant. When the dually infected subgroups (egg^+IgE^{hi}, egg^+IgE^{lo}, egg^-IgE^{hi}) were campared, distinct immune profiles emerged that suggested that some helminth infection phenotypes could favour HIV replication while others do not.

The fact that the results from the group of HIV-1 singly-infected individuals (egg^-/IgE^{lo}) were not particularly different from those of the groups with evidence of worm infection, and were almost similar to those of the egg^+IgE^{hi} group could be due to the complex dynamics of interactions between the two infections.

When comparing the egg^+/IgE^{hi} and egg^-/IgE^{lo} HIV uninfected groups, all measured variables, except the CD4:CD8 ratio, were lower (though not statistically significant except for eosinophils), in the former group. The relatively smaller numbers in the HIV negative subgroups could have influenced the power to obtain statistically significant differences. The fact that no such differences were noted in the HIV positive groups may suggest the probability that HIV-induced immunosuppression could be responsible for masking any differences in the latter. Firstly the HIV^+, egg^-/IgE^{lo} group had the lowest median $CD4^+$ counts (Table 3.4). Secondly, using $CD4^+$ counts independent of virus burden, similar percentages of participants in the egg^+/IgE^{hi} and egg^-/IgE^{lo} groups were severely immunocompromised (less than 0, $2x10^9$ cells/L $CD4^+$ counts) and were slightly higher in the egg^-/IgE^{lo} group. Furthermore, the proportions of individuals with higher virus burden (as determined by viral load >10^5 copies/ml) were also similar in the two groups (also slightly higher percentage in the egg^-/IgE^{lo} group). The individual with the maximum viral load was found in this group (Table 3.4). Furthermore, three individuals in the egg^-IgE^{lo} subgroup had virus burden exceeding 1 000 000 cpm. These observations may support the suggestion that HIV-induced immune suppression could obscure differences resulting from the effects of helminth infection and non-infection that may be present in the two groups.

Collorary to that suggestion may be an alternative, non-mutually exclusive explanation: The possibility that some participants within this group (without elevated IgE and no faecal helminth eggs) could be harbouring occult helminth infection as a result of being highly immunosuppressed was explored, in view of the unsanitary living conditions of this population. If indeed this group represents highly immuno-suppressed individuals, this could result in an inability to produce antibodies and excrete eggs.This is based on the reports that worm development and egg excretion are documented to be a function of a competent immune system: It has been shown in animal models that egg excretion, worm fecundity and larval size were smaller in immunocompromised mice compared to normal controls (Viney, 2002). Likewise HIV infected patients with lower $CD4^+$ counts and coinfected with *Schistosoma mansoni* excreted fewer eggs compared to HIV-uninfected schistosomiasis-patient controls (Karanja *et al.*, 1997). The authors ascribed these differences to the requirement for a competent immune system for egg excretion.

On the other hand, if indeed this group represents highly immuno-suppressed individuals, it would be expected that all cellular components (including eosinophils, total, T, $CD8^+$, NK and B lymphocytes would also be repressed, which was not the case. In addition, the median viral load for this group was lower than in the two groups who had elevated IgE and eosinophilia, although the individual with the maximum viral load was in this group. However, the fact that in the absence of HIV, there were differences in proportions of lymphocytes, wherein the egg^+/IgE^{hi} group had lower proportions of all lymphocyte subsets, compared to the group without evidence of helminth exposure suggests a confounder effect of HIV. The lower numbers of HIV negative groups limited the analysis. Based on these observations, the study hypothesis can neitherbe accepted or rejected, suggesting the need for increased numbers of controls for matching in future studies.

However, when the dually-infected subgroups were analysed, several observations suggest that certain immunological phenoytypes of helmninth infection may favour HIV replication, thus by inference lend support to the study hypothesis that helminthiasis might enhance virus replication. Firstly, typical helminth infection (as reflected by the egg^+/IgE^{hi} status) was accompanied by eosinophilia and showed a trend towards the reduction of all lymphocyte subsets when compared to the other three subgroups. This tendency was observed in both HIV positive and negative groups. This finding concurs with the report that chronic helminth infections in Ethiopian adults resulted in disruptions in peripheral T cell populations (Kalinkovinch *et* al., 1998). The observed decrease in lymphocyte populations among the egg^+/IgE^{hi} individuals in this study, when interpreted in light of the fact that that lymphocytes play a pivotal role in immune response to infection in general, could indirectly relate to a compromised immunological ability to respond to HIV. This would be in agreement with the study hypothesis.

A positive correlation between $CD4^+$ and $CD8^+$ counts was found in our study. Owing to the fact that the dynamics of total $CD8^+$ cell numbers during HIV infection are such that they remain high throughout all the phases of infection (primary, chronic infection and advanced (AIDS),and the fact that this is a cross sectional study and total $CD8^+$ cell numbers, not HIV-specific CD8+T cell responses were evaluated, the positive correlation observed in the present analysis is fitting.

Another observation in support of the study hypothesis is that high *Ascaris lumbricoides* IgE responders in the study population typically had associated eosinophilia particularly in the HIV negative group, and in the HIV+ this was observed only in the $egg^{+}IgE^{hi}$ group: both high IgE and eosinophilia are features of Th_2 responsiveness. Among the $HIV\text{-}1^{+}$ individuals, higher median viral load was found in both subgroups with high IgE. Upon grading the level of immunodeficiency by viral load, these groups also had the highest proportion of individuals with high virus burden. These findings suggest a relationship between Th_2 responses and an increased potential for HIV replication as reflected by high virus burden. In the HIV-1 uninfected group, hyper-IgE and eosinophilia showed a tendency towards relatively lower median $CD4^{+}$ counts (0.67 and 0.68 cells/ml compared to 0.75 and 0.76cells/ml respectively). By inference, it may be possible that if these people subsequently become HIV positive they would be less able to control the infection. Furthermore, eosinophils are documented to express CD4 molecules and have been shown to be infectable by HIV *in vitro* (Conway *et al*., 1992; Weller *et al*., 1995). Hypothetically, increased frequency of these cells increases the number of host cells receptive to the virus and therefore possibly sustains its replication cycle. It is noteworthy that while both high IgE responders in the HIV negative group had eosinophilia while in the HIV positive group, the $egg^{-}IgE^{hi}$ subgroup did not show an increase in these counts. One possible explanation could be the fact that this subgroup had the highest median viral load compared to all three subgroups. It could be related to virus-induced depletion of these cells that have been shown to be infectable by HIV.

The relationship between high IgE, eosinophilia and higher viral load found in the present study therefore support the study hypotheses suggesting that (i) Th_2 biased individuals fail to contain the HIV infection which is controlled by the Th_1 CTL response (ii) helminth infections favour HIV replication in certain helminth immune phenotypes.

It is noted that t, a significant inverse correlation between median $CD4^{+}$ counts and viral load was found for the entire HIV positive group,. However, although this relationship was strongest in both groups with high IgE, it was strong in all groups with some evidence of helminth infections and weakest in the group without evidence of worm exposure (egg^{-}/IgE^{lo}). High viral load and $CD4^{+}$ decline are prognostic markers of HIV disease progression. Such a relationship as observed in the high IgE groups (and all helminth-infected participants) in this study (Table 3.8a) would suggest a negative effect

of Th_2 responsiveness (and helminth infection) on HIV disease progression. However the sample size was very small to draw conclusions from this finding.

A distinct group of individuals was seen in the present section in both HIV+ and HIV- groups. Although these participants were infected by helminths as shown by the presence of faecal eggs, *Ascaris*-specific IgE levels were low (egg^+/IgE^{lo}). This group showed a tendency towards a better immune response to HIV: The observation of lower viral load (HIV^+ group), higher $CD4^+$ counts (HIV-group) and generally higher proportions of T- cell populations among the low *Ascaris lumbricoides* IgE responders in both HIV+ and HIV- participants suggests an advantage towards better control of HIV infection. Furthermore this group had the lowest proportion of individuals with immunodeficiency by both $CD4^+$ counts and viral load grading.

The fact that in both HIV^+ and HIV^-participants, low IgE groups who were stool egg positive had the lowest eosinophil counts (Tables 3.4 and 3.5) strongly suggests that these individuals lack the Th_2 mediators (IgE globulinaemia and eosinophilia) that are associated with protection against helminths (Maizels *et al.*, 2004). Positive stool egg without increased levels of Ascaris IgE in this group could theoretically suggest two possibilities. One could reflect a newly acquired infection before adaptive humoral responses are invoked, or could possibly imply their susceptibility to recurrent helminth infection in the absence of protective mechanisms conferred by IgE and eosinophils. The observations that this group had higher proportions of all lymphocyte subsets, suggest that their ability to respond to infections including HIV is comparatively competent e as indicated by higher median $CD4^+$ counts and lower viral loads and argues favourably for the hypothesis that this group represents a distinct Th_1 phenotype or lack of Th_2 bias. Furthermore, the grading of proportions of individuals with immunodeficiency consistently showed that this group had the lowest percentages of individuals with advanced HIV disease in terms of viral load and $CD4^+$ counts.

Indeed, according to Maizels and Yazdanbakhsh (2003) one of the three phenotypic outcomes of chronic exposure to helminths is a "modified Th_2" phenotype. According to these authors, the modified Th_2 individuals are characterised in part by low IgE and high IgG_4 antibody isotypes and low level of Th_1 cells. They are highly susceptible to asymptomatic, heavy helminth infections and are thus a reservoir for transmission.The

egg^+/IgE^{lo} status in the present study could be related to heavy infection in the absence of IgE secretion.

Total B and NK cells form part of the immune system's functional architecture, therefore it was necessary to quantify them in this study. No significant differences in these subsets were noted between the different subgroups, although the turnover rates of B cells were shown to be positively related to $CD4^+$ counts and viral load in a macaque-SIV infection model (De Boer *et al.*, 2003). In the present study no relationship could be established either and the results were not shown.

Finally, the study reveals data on phenotypes of HIV uninfected individuals residing in resource-limited settings in South Africa. The immunological markers that are relevant to HIV immune responses ($CD4^+$ and $CD8^+$) obtained from this study are lower than the CDC indices based on European reference ranges. However, the size of the sample representing presumably healthy individuals from this population was small therefore findings are interpreted with caution. The results are similar to locally generated reference ranges for Tygerberg hospital (P. Bouic, personal communication), which serve the community of Khayelitsha as well.

3.4. Conclusions

Four distinct patterns of results were observed:

1. No significant differences were observed when comparing the HIV positive egg^+/IgE^{hi} group with the HIV^+ group having neither faecal eggs nor elevated Ascaris IgE except for significant lower eosinophil counts, relatively higher individual numbers with a lower viral load and higher CD4+ counts in the latter. However, in the absence of HIV, a tendency to higher median counts in the egg^-/IgE^{lo} group with a significant difference in total lymphocytes and a lower trend in eosinophils were observed between these subgroups. The smaller sample size in the HIV negative groups possibly influenced the ability to reach statistical significance in differences among the other parameters. The results suggest a possibility of confounding by HIV. These outcomes suggest that the findings in the present section may not be adequate to allow the hypothesis to be rejected or accepted.

2. When dually-infected subgroups were compared, the presence of helminth stool eggs and high IgE (egg^+/IgE^{hi}) showed a tendency to reductions in all lymphocyte populations in both HIV infected and uninfected individuals. .

3. The two groups with high IgE (the egg^+/IgE^{hi} and egg^-/IgE^{hi} groups) typically had elevated Th_2 markers (high IgE and eosinophilia) and were associated with lower (but not statistically significant) $CD4^+$ counts in the HIV negative group and higher viral loads in the HIV infected group. Both subgroups had the higher percentages of individuals with higher viral load. In addition, there was the strongest inverse correlation between viral load and $CD4^+$ counts in these two subgroups. This has implications for HIV disease progression and helminth-induced Th_2 predominance.

4. Individuals with parasite eggs in stool but low Ascaris IgE (egg^+/IgE^{lo}) tended to have higher absolute numbers of all lymphocyte subsets in both HIV^- and HIV^+ groups and in particular significantly higher $CD4^+$ counts in the HIV^- and lower viral load in the HIV^+ groups. This subgroup of egg^+/IgE^{lo} individuals demonstrated features of a typical modified Th_2 helminth phenotype.

Analysis of dually infected subgroups' findings concur with the suggestions that helminth infections are associated with Th_2 predominance, (indicated by eosinophila and high IgE); cause disruption of peripheral lymphocyte populations and may favour HIV replication. The results further suggest the existence of a phenotypically different group of individuals who could be better able to control HIV replication (as shown by lower viral load and higher $CD4^+$ counts) but were susceptible to helminth infection as indicated by positive parasite eggs in stool.

References.

Adams VJ, Markus MB, Kwitshana ZL, *et al*. 2006. Recall of intestinal helminthiasis by HIV-infected South Africans and avoidance of possible misinterpretation of egg excretion in helminth/HIV co-infection analyses. *BMC Infectious Diseases.* **6 (88):** 1-8.

AIDS Education & Training Centers (AETC), 2006. HIV Classification: CDC and WHO Staging Systems. National Resource Center. http://www.aidsetc.org/aidsetc?page=cm-105_disease#t-2. Acessed 02-02 -2007.

Aina O, Dadik J, Charurat M, *et al.* 2005. Reference Values of CD4 T Lymphocytes in Human Immunodeficiency Virus-Negative Adult Nigerians. *Clinical Diagnostic and Laboratory Immunology.* **12(4):** 525-530.

Anastos K, Gange SJ, Lau B, *et al*. 2000. Association of race and gender with HIV-1 RNA levels and immunologic progression. *Journal of Acquired Immune Deficiency Syndrome.* **1;24** (3):218-26.

Anglaret X, Diagbouga S, Mortier E, *et al*. 1997. $CD4^+$ T- lymphocyte counts in HIV infection: are European standards applicable to African patients? *J Acquir Immune Defic Syndr Hum Retrovirol.* **14 (4):**361-367.

Bagnarelli P, Valenza A, Menzo S, Manzin A, Scalise G, Varaldo PE, Clementi M. 1994. Dynamics of molecular parameters of human immunodeficiency virus type 1 activity *in vivo*. *Journal of Virology:* **68 (4):** 2495-2502**.**

BDBiosciences.2004.Flowcytometry.
http://www.bdbiosciences.com/immunocytometry_systems/ Accessed 03-24 -2005.

Betts MR, Ambrozak DR, Douek DC *et al*. 2001. Analysis of total human immunodeficiency virus (HIV)-specific $CD4^+$ and $CD8^+$ T –cell responses: Relationship to viral load in untreated HIV infection. *Journal of Virology***. 75 (24):**11983-11991.

Brown AE, Malone JD, Zhou SYJ, Lane JR, Hawkes CA. 1997. Human immuno-deficiency virus RNA in US adults: a comparison based upon race and ethnicity. *Journal of Infectious Disease.* **176:**794-797.

Brown M, Mawa PA, Kaleebu P, Elliott M. 2006. Helminths and HIV infection: epidemiological observations on immunological hypotheses. *Parasite Immunology.* **28 (11):** 613–623.

Bundy DAP. 1990. Human behavior and the epidemiology of helminth infections: the role of behavior in exposure to infection In. Parasitism and host behaviour -Barnard CJ, Behnke JM. (eds) pp 264-299. London. Taylor and Francis.

Centers for Disease Control and Prevention. (CDC). 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR Recomm Rep*. 1992 Dec 18; **41(RR-17):**1-19.

CDC.1997 Revised Guidelines for Performing $CD4^+$ T-Cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV) *MMWR* **46(RR-2);** 1-29.

CDC Laboratory Procedure Manual. $CD4^+$ T-lymphocytes and $CD8^+$T cells NHANES 2003-2004. Immunophenotyping Fresh-Frozen Whole Blood Specimens Using the BD TruCountMethod.
http://www.cdc.gov/nchs/data/nhanes/nhanes/0304/103cmetCD4+CD8+ Accessed 02-25-2008

Conway B, Baskar P, Bechtel LJ, *et al*. 1992. Eosinophils as host cells for HIV-1. *Archives of Virology.***127 (1-4):**373-377.

Davey HM, Kell DB. 1996. Flow Cytomerty and Cell sorting of heterogeneous microbial populations: The importance of single cell analyses. *Microbiological Reviews.***60 (4)** 641-696.

De Boer RJ, Mohri H, Ho DD, Perelson AS. 2003. Turnover rates of B cells, T cells and NK cells in Simian Immunodeficiency vurus –infected and uninfected Rhesus Macaques**.** *The Journal of Immunology.* **170:**2479-2487.

De Souza MS, Karnasuta C, Brown AE, *et al.* 2000. A comparative study of the impact of HIV on Natural Killer cell number and function in Thais and North Americans. *AIDS Research and Human Retroviruses.***16(11):** 1061-1066.

Else KJ, Grencis RK. 1991. Cellular immune responses to the murine nematode parasite *Trichuris muris.* 1. Differential cytokine production during acute or chronic infection. *Immunology.* **72:** 508-513.

Else KJ, Grencis RK. 1996. Antibody-independent effector mechanisms in resistance to the intestinal nematode parasite *Trichuris muris*. *Infection and Immunity.* **64 (8):**2950-2954.

Giorgi J. 1993. Characterisation of T lymphocyte subset alterations by flow cytometry in HIV disease. *Annals of the New Academy of Sciences.* Clinical flow cytometry. **677:**126.

Hu PF, Hultin LE, Hultin P, *et al*. 1995. Natural killer cell immunodeficiency in HIV is manifest by profoundly decreased numbers of $CD16^{+}CD56^{+}$ cells and expansion of a population of CD16 dim CD56- cells with low lytic activity. *Journal of Acquired Immunodeficiency Syndrome and Human Retroviruses.* **10 (3):** 331-340.

Jin X, Bauer DE, Tuttleton SE *et al*. 1999. Dramatic rise in plasma viremia after $CD8^{+}$ Tcell depletion in Simian Immunodeficiency virus-infected macaques**.** *Journal of Experimental Medicine.* **189 (6)** 991-998.

Kalinkovich A, Weisman Z, Greenberg Z, *et al.* 1998. Decreased CD4 and increased CD8 counts with T cell activation is associated with chronic helminth infection. *Clin. Exp. Immunol.* **114:** 414-421.

Karanja DMS, Colley DG, Nahlen BL, *et al.* 1997. Studies on Schistosomiasis in Western Kenya : I. Evidence for immune-facilitated excretion of schistosome eggs from

patients with *Schistosoma mansoni* and Human Immunodeficiency Virus co-infections. *American Journal of Tropical Medicine and Hygiene.* **56 (5) :**515-521.

Katzenstein DA, Latif AS, Grace SA, *et al.* 1990. Clinical and laboratory characteristics of HIV-1 infection in Zimbabwe. *Journal of Acquired Immune Deficiency Syndrome.* **3 (7):** 701-707.

Kestens L, Brattegaard K, Adjorlolo G, *et* al. 1992. Immunological comaparison of HIV-1 and HIV-2 and dually reactive women living in Abidjan, Cote d'Ivore. *AIDS.* **6:**803-807.

Kottilil S, Shin K, Planta M, *et al.* 2004. Expression of chemokine and inhibitory receptors on Natural Killer cells: Effect of Immune activation and HIV viraemia*. The Journal of Infectious Diseases.* **189 (3):** 1193-1198.

Koup RA, Safrit JT, Cao Y, *et al.* 1994. Temporal association of cellular immune responses with the initial control of viremia in primary HIV-1 syndrome. *Journal of Virology.* **68:**4650-4655.

Kunkl A. 1997. Flow cytometric immunophenotyping of HIV infected participants and quality control**.** In: The Purdue Cytometry CD-ROM Volume 3, J. Parker, C. Stewart, Guest Eds., J.Paul Robinson, Publisher. Purdue University, West Lafayette, 1997, ISBN 1-890473-02-2

Ledergerber B and the PLATO Collaboration. 2004. Predictors of trend in CD4-positive T-cell count and mortality among HIV-1-infected individuals with virological failure to all three antiretroviral-drug classes. *The Lancet.* **364 (9428):** 51-62.

Lederman MA, Kalish, L A, Asmuth D, Fiebig E, Mileno M, Busch MP. 2000. `Modeling' relationships among HIV-1 replication, immune activation and CD4+ T-cell losses using adjusted correlative analyses. *AIDS.* **14(8):** 951-958

Le Guenno BM, Barabe P, Griffet PA, Guiraud M, *et al.* 1991. HIV-2 and HIV-1 AIDS cases in Senegal: clinical patterns and imminological perturbations. *Journal of Acquired Immune Deficiency Syndrome***. 4 (4):** 421-427.

Levin A, Brubuker G, Shao JS, *et al.* 1996. Determination of T-lymphocyte subsets on site in rural Tanzania: results in HIV-1 infected and uninfected individuals. *International Journal of STD and AIDS.* **7:**288-291.

Lugada ES, Mermin J, Kaharuza F, *et al.* 2004. Population-based Haematologic and immunologic reference values for a healthy Ugandan population. *Clinical and Diagnostic Laboratory Immunology.* **11 (1):** 29-34.

Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. 2004. Helminth parasites-Masters of regulation. *Immunology Reviews.* **201:** 89-116**.**

Maizels RM, Yazdanbakhsh M. 2003. Immune regulation by helminth parasites: Cellular and molecular mechanisms. *Nature Reviews Immunology.* **3:** 733-744.

Mavillo D, Lombardo G, Benjamin J**,** *et al.* 2005. Characterisation of CD56-/CD16^{+} natural killer cells: A highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proceedings of the National Academy of Sciences of the USA.* **(102) 8**:2886-2891.

May RM, Anderson RM. 1979. Population biology of infectious diseases. Part II. *Nature.* **280**: 455-461.

Meier UC, Owen RE, Taylor E, *et al.* 2005. Shared alterations in NK cell frequency, phenotype and function in chronic human immunodeficiency virus and hepatitis C virus infections. *Journal of Virology***. 79 (19):**12365-12374**.**

Mellors JW, Rinaldo CR Jr, Gupta P, *et al.*, 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science.* **272 (5265):**1124-1125

Mellors JW, Muñoz A, Giorgi JV, *et al.*, 1997. Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Annals of Internal Medicine.***15126 (12):**983-985.

Metzner KJ, Jin X, Lee VF, *et al.,* 2000. Effects of *In vivo* CD8+ T cell depletion on virus replication in Rhesus Macaques immunized with a live, attenuated Simian Immunodeficiency Virus vaccine. *Journal of Experimental Medicine.* **191 (11):** 1921-1931.

Nelson R. 2006. Viral loads not indicative of CD4 decline in HIV patients. *The Lancet.* **6:** 689.

Nolte FS. 1999. Impact of viral load testing on patient care. *Archives of Pathology and Laboratory Medicine.* **123:**1011-1014.

Pollack S, Fuad B, Etzioni A. 1993. CD4 T-lymphocytopenia without opportunistic infections in HIV-seronegative Ethiopian immigrants to Israel .*The Lancet.* **342:** 8862, 50.

Rizzardini G, Trabattoni D, Saresella M, *et al*. 1998. Immune activation in HIV-infected African individuals. Italian-Ugandan AIDS cooperation program. *AIDS.* **12(18):**2387-2396.

Saul J, Erwin J, Sabin CA, Kulasegaram R, Peters BS. 2001. The relationship between ethnicity, sex, risk group and virus load in human immunodeficiency virus type 1 antiretroviral-naïve patients. *Journal of Infectious Disease.* **183:**1518-1521.

Smith PR, Sarner L, Murphy M *et al*. 2003. Ethnicity and discordance in plamsa HIV-1 RNA viral load and CD4+ lymphocytes count in a cohort of HIV-1 infected individuals. *Journal of Clinical Virology*.**26 (1):** 101-107.

Soltys J, Goyal PK, Wakelin D. 1999. Cellular immune responses in mice infected with the intestinal nematode *Trichuris muris*. *Experimental Parasitology.* **92:**40-47.

Swindells S, Cobos DG, Lee N, *et al*. 2002. Racial/ethnic differences in CD4 T cell count and viral load at presentation for medical care and in follow-up after HIV-1 infection *AIDS.* **16 (13):** 1832-1834

Tugume BS, Piwowar EM, Lutalo T, *et al*. 1995. Haematological reference ranges among healthy Ugandans. *Clinical Diagnostic and Laboratory Immunology* **2:** 233-235.

Urassa WK, Mbena EM, Swaib AB, Gainesc H, Mhalua FS, Biberfeld G. 2003. Lymphocyte subset enumeration in HIV seronegative and HIV-1 seropositive adults in Dar es Salaam, Tanzania: determination of reference values in males and females and comparison of two flow cytometric methods. *Journal of Immunological Methods*. **277(1-2):** 65-74

van Gool T, Weijts R, Lommerse E, Mank TG. 2003. Triple faeces test: An effective tool for detection of intestinal parasites in routine clinical practice. *European Journal of Clinical Microbiology & Infectious Diseases*. **22** (5): 284-290, DOI: 10.1007/s10096-003-0919-1 Accessed 11 Octber 2007.

Viney M. 2002. Developing worms need the host immune system*. Trends in Parasitology.* **18 (2):** 57.

Walker JM. 1998. Flow cytometry protocols. In: Jaroszeski MJ, Heller R.eds Methods in Molecular Biology.Vol 91. Humana Press.Totowa, NJ.

Wei X, Ghosh SK, Taylor ME, *et al.* 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature.* **373:**117-22.

Weller PF, Marshall WL, Lucey DR, *et al*. 1995. Infection, apoptosis, and killing of mature human eosinophils by Human Immunodeficiency Virus-1. *American Journal of Respiratory Cell & Molecular Biology.* **13(5):**610-620.

Whittle H, Egboga A, Todd J *et* al. 1993. Immunological responses of Gambians in relation to clinical stage of HIV-2 disease. *Clinical Experimental Immunology.* **93:**45-50.

Wolday D, Tsegaye A, Messele T. 2002. Low absolute CD4 counts in Ethiopians. *Ethiopian Medical Journal.* **(40) S1:**11. 6

World Heatlh Organisation. 2005. Interim WHO clinical staging of HIV/AIDS and HIV/AIDS case definitions for surveillance for persons aged 15 years and above positive for HIVantibody test- African Region. *Reference number: WHO/HIV/2005.02*

Chapter 4

Immunophenotyping of cell surface activation markers on T lymphocyte subsets of HIV positive and negative subgroups with or without helminth co-infection.

4.1 Introduction

Both HIV and helminth infections are chronic and both induce widespread immune activation (Lawn *et al.*, 2001; Borkow and Bentwich, 2004). It has been shown that immune activation contributes significantly to drive HIV disease progression. Leng *et al.*, (2001) demonstrated that immune activation correlated better with $CD4^{+}$ cell decline than levels of HIV virus in plasma. Similarly, Hazernberg and colleagues (2003) showed that continuous immune hyperactivation correlates with AIDS progression. Even earlier workers illustrated this positive association between T cell activation and HIV infection advancement to AIDS (Mahalingam *et al.*, 1993; Giorgi *et al.*, 1999). The cellular, molecular and biological mechanisms by which immune activation aggravates HIV pathogenesis have been thoroughly reviewed by Lawn *et al.* (2001) and Borkow and Bentwich (2004) and were described briefly in Chapter 1.

Helminths have been widely implicated to cause extensive immune activation in developing countries (Bentwitch *et al.*, 1995, 1996, 1997, 1999; Clerici *et al.*, 2000; Borkow and Bentwich, 2004). A direct causal link between immune activation and helminth infection was demonstrated in the study by Kassu *et al.*, 2003, in which treatment of parasitic infections reduced the level of immune activation among Ethiopians.

Immune activation is accompanied by increased expression of a variety of cellular components. Typically, expression of the major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells is central to effective antigen processing for presentation. As such, expression of the human leukocyte antigen-D related (HLA-DR) molecule is a classic marker of activation. Increased expression of this marker molecule has been associated with helminth-induced chronic activation of the

immune system. HLA-DR expression was significantly increased in all T cell subsets of newly-arrived Ethiopian immigrants to Israel who were infected with helminths (Kalinkovich *et al.*, 1998). In HIV infection as well, HLA-DR is upregulated as T cell activation is a prominent feature of HIV (Hazernberg *et al.*, 2003; Shang *et al.*, 2005).

Likewise, expression of chemokine receptors is strongly linked to activation. The G-protein coupled receptor CCR5 for the chemokines RANTES, MIP-1α and MIP-1β that are involved in lymphocyte recruitment and activation, is reported to be elevated in chronic infections and particularly documented in chronic helminthiasis (Kalinkovinch *et al.*, 1999; 2001 Clerici *et al.*, 2000) as well as during HIV infection (Ostrowski *et al.*, 1998, Shalekoff *et al.,* Iqbal *et al.*, 2005, Shang *et al.,* 2005). CCR5 is regulated by cytokines: IL-10 has been shown to upregulate its expression (Bleul *et al.*, 1997; Sozzani *et al.*, 1998) and is predominantly expressed on memory cells (Bleul *et al.*, 1997). Helminths are documented to increase both the memory cell pool ($CD45RO^+$) (Kalinkovinch *et al.*, 1998) and IL-10 production (Borkow and Bentwich, 2004). It is therefore expected that under conditions of continuous helminth exposure, CCR5 would be elevated.

Other cell surface markers associated with chronic infection include the IL-2 receptor α chain (CD25). $CD25^+CD4^+$ cells were shown to suppress Th_1 responses in mice infected with schistosomes and thus inferred to be responsible for Th_2 polarisation in helminth infection (McKee and Pearce, 2004). The transferrin receptor (CD71) is also expressed by all proliferating, activated cells (Babusíková *et al.* 1999). It is an early activation marker, thus its increased expression indicates active proliferation and activation.

In HIV infection, increased CD38 expression is associated with disease progression, with higher levels being indicative of poor prognosis (Kunkl, 1997; Shang *et al.*, 2005). It is suggested that approximately 20 % of $CD8^+$ cells express CD38 in healthy individuals and in HIV infection, levels above 50% $CD8^+$ $CD38^+$ signify extensively poor prognosis (Kunkl, 1997). Increased CD38 expression, in addition to signifying activation, would thus also indirectly assess the HIV disease progression.

Since the two chronic infections (HIV and helminths) result in sustained immune activation, hypothetically, upregulation of activation markers on peripheral lymphocytes

would be more pronounced in co-infected individuals, and their immune responses to HIV would thus be attenuated.

This chapter characterises the activation profile of HIV-1 infected and uninfected participants with or without intestinal parasite co-infection. To determine whether there are significant differences in the activation status among HIV positive individuals with evidence of exposure to or with active enteric helminth co-infection relative to those without helminths, the expression of activation markers on their T cell membranes were compared. In order to do this, cryopreserved PBMC's were thawed, washed, stained with monoclonal antibody-fluorochrome conjugates and analysed by 4-colour flow cytometry. The percentages of cells expressing the activation markers CCR5, CD25, HLA-DR, CD38 and CD71 were determined on $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes. HIV negative participants with or without helminth infections were included for comparisons by HIV status.

4.2 Methods

4.2.1. Selection of Participants

Owing to budgetary constraints against the costs of the laboratory tests, a limited number of these individuals could be sampled to undertake the multiple assays for this pilot hypothesis testing study, hence only 40 HIV positive (ten in each of the four subgroups) and all the twenty-two HIV negative, eligible individuals were selected. The 40 HIV^+ participants were randomly selected from 124 participants who had complete data sets in terms of stool egg results, Ascaris-specific IgE (for proper classification of helminth infection status) and lymphocyte phenotypes as described in section 2.3 Figure 2.1 legend **"**Those eligible donated blood and stool samples. Helminth status was determined by both colproscopy and serology and lymphocyte immunophenotypes done. Those with incomplete data for helminth status classification were further excluded. A subset of the eligible participants was sequentially selected (every second individual) using study identification numbers for further immunological analysis. *Among the HIV^- eligible participants, 23 did not submit the complete set of samples thus the remaining 22 were included by default. The selected individuals were to have adequate cell numbers to allow multiple testing of their PBMCs.

4.2.2. Collection and Cryopreservation of PBMCs

Blood specimens were collected into Cell Preparation Tubes (CPT^{TM}) vacutainer tubes (BD) with Sodium Heparin (120 Units in 1 ml PBS) and submitted to the laboratory within 3 hours (required to be less than 24 hours old).

All the separation steps, except centrifugation, were performed under a laminar flow hood under aseptic conditions. For each patient sample, two of 2ml sterile Corning cryogenic vials (Adcock Ingram) were pre-labeled with patient code, study title, date and visit number. The CPT tubes were coded, labeled and centrifuged for 20 minutes at 1600^{+}-100*g* (2800rpm) at 22°C. (NB: centrifugation at 4-8°C inhibits collection of cells at interface). The tubes were inverted 5-10 times to mix plasma and cells. Then the plasma and white cells were aseptically transferred to sterile, polypropylene 15ml conical centrifuge tubes (Lasec). The cells were sedimented by centrifugation at 400*g* for 10 minutes at 22°C. The supernatant plasma containing platelets was transferred to a new sterile centrifuge tube and further centrifuged at 1100*g* for 10 minutes to pellet the platelets. Plasma (free of platelets) was then frozen at -80ºC. Sterile PBS pH 7,2 (Sigma) was added to the 14 ml mark of the conical centrifuge tube containing the pelleted cells and the tubes were capped and inverted five times to mix and wash thoroughly. The tubes were then centrifuged at 500^{+}-50*g* at 22°C for 10 minutes. The supernatant was decanted without disturbing the cells. Cell pellets were resuspended in 14 ml sterile PBS, thoroughly mixed and the wash/centrifugation steps repeated once more. All excess PBS was removed and cold 0,5ml fetal calf serum (FCS) (Biowhittakker) was added to each tube to resuspend the cells which were then cooled on ice. A 20% v/v solution of Dimethylsulfoxide (DMSO) (Sigma) in RPMI 1640 culture medium with glutamine (Sigma) without penicillin and streptomycin was freshly prepared and cooled on ice. Two hundred and fifty microliters of the cell suspension on FCS were transferred to each of the two appropriately labeled 2ml cryogenic vials. Two hundred and fifty microliters of cold 20% DMSO in RPMI were added dropwise to each of the
250 µl cells suspended in FCS (NB: The DMSO always was added to the cells and not the other way round) to give a final 10% DMSO concentration. The cryogenic vials were wrapped in several layers of paper towel and frozen at -80°C for at least 24 hours. The cryotubes were then transferred into Liquid Nitrogen (LN) storage tanks (they could be stored at -80°C for one year or longer in liquid nitrogen). Datasheets describing each

sample's identity, position in the storage boxes and liquid nitrogen tank were logged and kept safely.

4.2.3 Retrieval and Thawing of Frozen PBMCs

Forty baseline samples of PBMCs from HIV-1 positive participants and 22 from HIV negative controls (each in duplicates of 2ml cryovials each) were retrieved from cryopreservation. The respective positions of the samples were located from the storage data sheets. An adequate volume of liquid nitrogen was transferred to the holding Dewar. Samples were then quickly removed from the LN storage tank and immediately transferred to the temporal holder containing liquid nitrogen until ready to be thawed.

The cryovials were immediately immersed into a 37ºC water bath and the cells were gently mixed while thawing. Once the last lump of solid ice dissolved, the cells were transferred into a 15ml Falcon polypropylene tube. Using a 10ml sterile, disposable pipette, cold, 10ml of RPMI with 10% FCS (both from Sigma) was added drop-wise to avoid rapid osmotic shock to the cells. The suspension was centrifuged for 10 min at 400*g* at 22ºC) and all the supernatant containing DMSO removed aseptically. The pelleted cells were suspended in 10ml RPMI and washed once more by centrifugation as above. The cells were resuspended in 1ml of culture medium RPMI-1640 with glutamax (Gibco), containing 10% human male AB serum, US origin, heat-inactivated (Gibco), 2-mercaptoethanol (50µM), penicillin 100U/ml and streptomycin 1000ug/ml and counted.

4.2.4 Enumeration of PBMCs

Cell counts were performed by filling the Neubauer counting chamber with 10µl of cell suspension and the cells enumerated. A phase-contrast microscope was used to simultaneously discriminate between viable and non-viable cells. All viable and non viable cells were counted. For each sample no more than 10% dead cells were accepted for further experiments. The total volume of all cell suspensions was adjusted to 5.5 $x10^6$ cells/ml, so that 5 ml was used for initiation of cultures (for studies in Chapter 5) and 0.5 ml used for surface staining of activation markers (section 4.2.4).

4.2.5 Surface Staining and Flow Cytometric Measurements of Activation Markers

To determine the *ex-vivo* activation status of participants, their PBMCs were labeled with directly conjugated monoclonal antibodies to surface markers of activation and measured by flow cytometry.

4.2.5.1 Methodology and Modification

The procedure described in the BD Biosciences/Pharmingen Product Catalog, Technical Resources (2003) was undertaken and modified for v-bottom plates as described in section 3.2.1.2 in Chapter 3 above. Further modifications were that the cells were resuspended in complete RPMI instead of staining buffer before staining. Different volumes of monoclonal antibodies (5, 10, 15 and 20 μl) were investigated and all yielded equivalent staining, thus 5 μl were used in all staining procedures.

4.2.5.2 Procedure

All monoclonal antibodies (α) were purchased from BD already directly conjugated to the fluorochromes as listed in Table 4.1. Forty baseline samples of PBMCs from HIV-1 positive participants and 22 from HIV negative controls (in duplicate 2ml cryovials each) were retrieved. For each sample, 5 wells of the 96 well, v-bottom microtiter plate were designated for different antibody-fluorochrome conjugates as tabulated in Table 4.1.

Table 4.1 Monoclonal antibody-fluorochrome combinations and microplate-well designation for samples.

Combinations of monoclonal antibody-fluorochrome conjugates were added to each designated well of a microtiter plate for staining PBMC samples. Each sample was allocated 5 wells for labeling the surface markers CD3, CD4, CD8 and the activation markers CCR5, CD25, HLA-DR, CD38 and CD71.

Well number 1	Well no. 2	Well no. 3	Well no. 4	Well no.5
CD3 PerCP	CD3 PerCP	CD3 PerCP	CD3PerCP	CD3 PerCP
CD4 APC	CD4 APC	CD4 APC	CD8 FITC	CD4 APC
CD8 FITC	CD8 FITC	CD8 PE	CD38 PE	CD8 PE
CCR5 PE	CD25 PE	HLA-DR FITC	*CD28APC	CD71 FITC

* Results for CD28 staining were not reproducible for all experiments thefore excluded for all analyses.

Five microliters of each antibody conjugate was carefully pipetted to the bottom of each designated well. One hundred microliters of cell suspensions (from section 4.2.3 above), containing $1x10^5$ cells/ml in complete RPMI with glutamax, human AB serum (Gibco) and antibiotics were added to each corresponding well (five wells per sample) and mixed. The solutions were covered and mixed by gently shaking in a multiwell plate shaker (Sigma) at 1000 rpm for 30 seconds. The plates were incubated in the dark on ice for 45 minutes. The cells and antibody conjugates were pelleted by centrifugation at 2500 rpm for 3 min at 4ºC and the supernatant was aspirated by vacuum-suction. One hundred and eighty microliters of filtered wash buffer (1x PBS containing 0,1% sodium azide-(NaN_3), pH 7,2) was added to each well to resuspend the pellets and centrifuged as above. The wash-centrifugation step was repeated one more time. The stained cell pellets were resuspended in 180µL of fixative (4% v/v formaldehyde in filtered PBS) and mixed thoroughly. The samples were then quantitatively transferred to 12x75mm BD FACS™ tubes containing 450 µl of 4 % v/v formaldehyde in filtered PBS. These could be kept at 4º C in the dark for up to 24 hours or analysed immediately on the FACS Calibur™ flow cytometer. (NB: wells containing unstained cells from HIV-1 positive and negative individuals, cells labelled with isotype control antibodies as well as separate cells labeled with individual and combined antibody-flourochrome conjugates were included in each experiment for threshold and PMT settings, autoflourescence control, non-specific staining, negative population setting and spectral overlap colour compensation).

4.2.5 Flow Cytometry: Acquisition and analysis

The daily instrument standardization and maintenance was performed. Using the Cellquest software for four colour analysis, the unstained cells were first run through the instrument for the autoflourescence control and isotype controls for non-specific binding of monoclonal antibodies. Forward and side scatter detector PMT voltage and Amp gain settings were also adjusted using the unstained cells tube. The threshold value was set at 180 on forward scatter to exclude debris. Then tubes containing cells labeled with one and two antibodies were used to compensate for spectral overlap in the four channels between FL1 and FL2, FL1 and FL3, FL2 and FL3, and FL3 and FL4. Cells stained with isotype controls were used to exclude non-specific binding of antibodies. The following

isotype controls were included: IgG_1k and IgG_2a FITC; IgG_1k and IgG_2a PE; IgG_1k PerCP and IgG_2a APC. Further, unstained cells from a presumably healthy (HIV and helminth negative) individual from the same community were also used to set the negative population marker. The cells from the study population appeared to have increased autofluorescence and/non specific staining than isotype controls and normal laboratory donor cells. In addition, the normal laboratory donor cells were obtained from individuals who were either of a different ethnicity, thus for the study, appropriate controls were selected from the study population individuals.

The samples were then run on Cellquest software. A minimum of 10 000 events were acquired for each sample tube in list mode. Various gating strategies were attempted to eventually improve the recovery of all lymphocytes from the samples for optimal measurement of surface markers. The final gating strategy is described below:

4.2.6 Gating strategy for lymphocyte subsets

Lymphocytes were gated on side and forward light scatter properties as in region 1 (R1) of Figure 4.1. T lymphocytes were further differentiated by CD3 expression (R2) on the side scatter vs CD3 plot to improve discrimination of lymphocytes. The T lymphocyte subpopulations were separated on the $CD4^+$ vs $CD8^+$ dot plot, as R3 and R4 respectively to create a stringent gate that would facilitate exclusion of double positive ($CD4^+$ $CD8^+$) cells. Finally all $CD4^+$ (gated on R1, R2 and R3) and $CD8^+$ (gated on R1, R2 and R4) expressing the respective activation markers were individually plotted. All analyses were done using this gating strategy. Quadrant markers were set using cells from an HIV and parasites negative individual from the same population as the use of laboratory healthy controls proved unsuitable. Use of density as well as dot plots yielded similar results.

Membrane expression of T cell activation markers was determined using this four colour flow cytometry method. Percentages of each T cell subset expressing the relevant marker were established on gated lymphocytes.

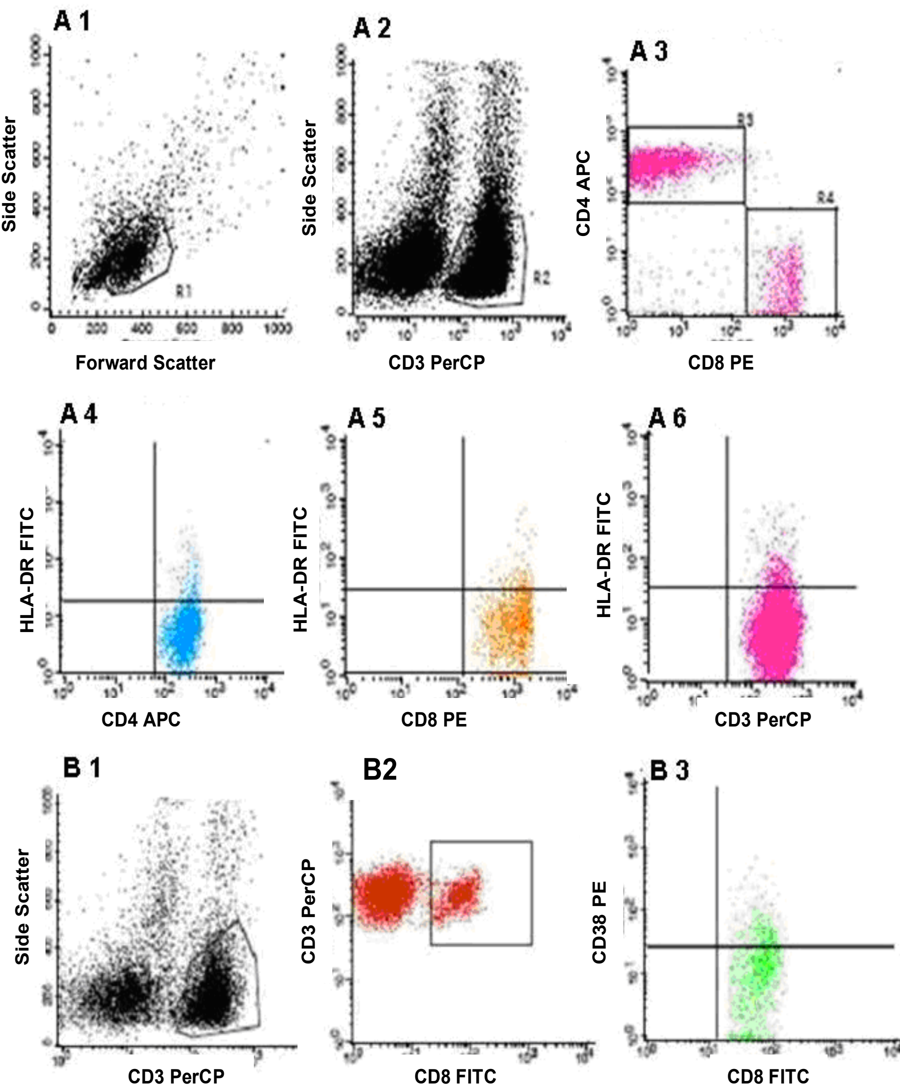


Figure 4.1. **Gating strategy to identify percentages of CD3, CD4 and CD8 cells expressing activation markers (HLA-DR in this illustration A1-A6).**

PBMCs were labeled with monoclonal antibodies to CD3, CD4, CD8 and HLA-DR conjugated to PerCP, APC, PE and FITC fluorochromes respectively. The resulting fluorescence was measured by flow cytometry. A1 A Lymphocyte gate based on light scatter properties on the side scatter versus forward scatter dot plot was set in region 1 (R1) as lowest side scatter and highest forward scatter. A2 T lymphocytes are differentiated by CD3 expression (R2) on the side scatter versus CD3 dot plot, which facilitates the differentiation (in A3) of $CD4^+$ and $CD8^+$ cells in regions R3 and R4 respectively. The percentages of each T lymphocyte population expressing the particular surface markers on their membranes were then measured in A4, A5 and A6. CD38 expression on $CD8^+$ was gated separately as illustrated in (B). A Lymphocyte gate was based on light scatter and CD3 expression properties (R1) in B1. $CD8^+$ lymphocytes were gated on the $CD3^+$ versus $CD8^+$ plot in B2. The percentages of $CD8^+$T lymphocytes expressing CD38 on their membranes were then measured on the CD8 versus CD38 plot (B3). All positive cells appear on the upper right quadrants in A4, A5, A6 and B3. Each dot represents an event in all dot plots.

4.3 Results

Median percentage cells expressing the activation markers were compared by Kruskal Wallis ANOVA within subgroups of HIV^+ and HIV^- individuals with and without helminth co-infection. Differences were considered significant at $p \leq 0.05$.

4.3.1. Summary activation profiles in HIV^+ and HIV^- groups.

Tables 4.2 and 4.3 summarise the median, minimum and maximum values of percentage expression of activation markers for each T cell subset in HIV^+ and HIV^- groups respectively. Generally, marked differences in median values for expression of surface markers were observed between the HIV positive and negative groups (Tables 4.2 and 4.3 and Figure 4.2).

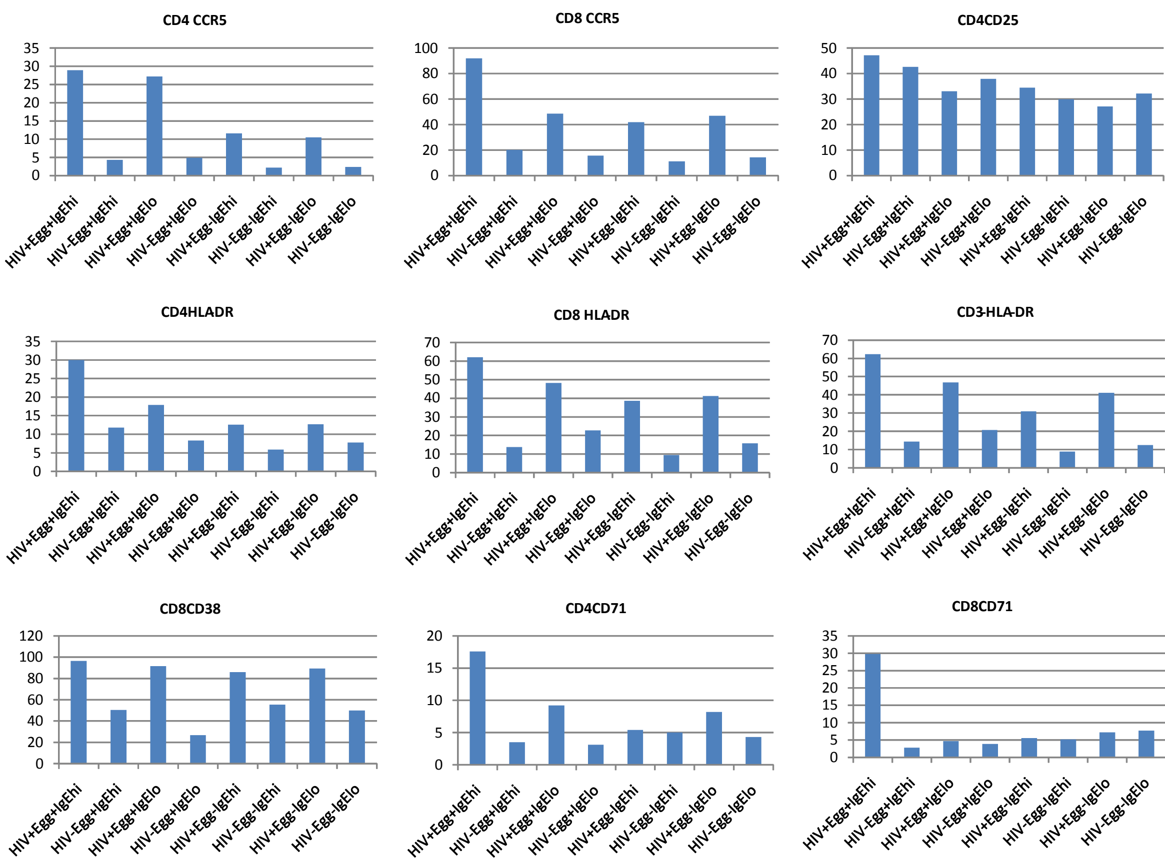


Figure 4.2. Median percentage expression of surface activation markers on T lymphocyte subsets in HIV+ and HIV- subgroups with or without helminth co-infection. PBMCs were stained with fluorochrome-conjugated monoclonal antibodies to surface lymphotype and activation marker molecules and the proportions of T cell subsets expressing the particular markers quantitated by flow cytometry. Numbers on the Y axis represent percentages and each bar represents the median percentages of each lymphocyte type expressing the respective marker in each subgroup. It is noted that the HIV+egg+IgEhi subgroup expressed very high levels of activation markers compared to all subgroups.

Table 4.2 Median percentage expression of surface activation markers on T lymphocyte subsets in HIV infected groups with or without helminth co-infection.

Subgroups were classified according to presence or absence of faecal eggs and high or low Ascaris IgE. Cryopreseved PBMCs were stained with fluorochrome-conjugated monoclonal antibodies to surface lymphotype and activation marker molecules and the proportions of T cell subsets expressing the particular markers quantitated by flow cytometry. Values represent median percentages of each lymphocyte type expressing the respective marker in each subgroup. Table 4.4 (page 139) shows the p values of all significant interactions.

Group	Marker	CD4-CCR5	CD8 CCR5	CD4-CD25	CD4-DR	CD8-DR	CD3-DR	CD8CD38	CD4-CD71	CD8 CD71	CD4	Viral load
Egg^{+} IgE^{hi} (n=10)	**Median**	**28.9**	**91.8**	**47.2**	**30.0**	**62.1**	**62.3**	**96.5**	**17.6**	**29.8**	**0.27**	**101006.5**
	Min	13.0	76.6	38.2	11.7	45.9	44.9	92.2	4.5	15.6	.028	7563
	Max	61.6	98.4	60.4	57.9	79.7	83.9	99.5	47.7	57.0	.645	1013265
Egg^{+} IgE^{lo} (n=10)	**Median**	**27.2**	**48.6**	**33.1**	**17.9**	**48.2**	**46.8**	**91.5**	**9.2**	**4.7**	**0.4**	**77 290**
	Min	9.6	23.8	27.4	7.7	26.9	23.5	81.3	3.5	2.0	.051	1818
	Max	33.2	65.8	47.9	37.2	80.5	83.5	99.8	15.8	9.6	.906	307768
Egg^{-} IgE^{hi} (n=10)	**Median**	**11.6**	**41.9**	**34.5**	**12.6**	**38.6**	**31.0**	**86.0**	**5.4**	**5.6**	**0.44**	**42274**
	Min	5.7	20.5	15.0	7.7	20.9	16.9	66.1	3.2	3.7	.16	170
	Max	26.2	57.4	60.4	42.6	74.7	66.2	97.5	14.6	11.0	.99	222 371
Egg^{-} IgE^{lo} (n=10)	**Median**	**10.5**	**47.0**	**27.1**	**12.7**	**41.3**	**41.1**	**89.3**	**8.2**	**7.2**	**.22**	**4234**
	Min	4.8	42.6	20.3	5.1	13.7	15.0	82.0	5.7	3.0	.117	509
	Max	23.6	53.0	42.7	27.5	56.5	56.6	98.3	16.6	19.8	.3	262962

Egg^{+} IgE^{hi} : faecal helminth eggs and high serum *Ascaris lumbricoides*-specific IgE (high IgE >0.35KU/L);
Egg^{+} IgE^{lo} :faecal helminth eggs and low serum *Ascaris lumbricoides*-specific IgE; (low IgE <0.35KU/L)
Egg^{-} IgE^{hi} :no faecal helminth eggs and high serum *Ascaris lumbricoides*-specific IgE;
Egg^{-} IgE^{lo}: no faecal helminth eggs and low serum *Ascaris lumbricoides*-specific IgE.

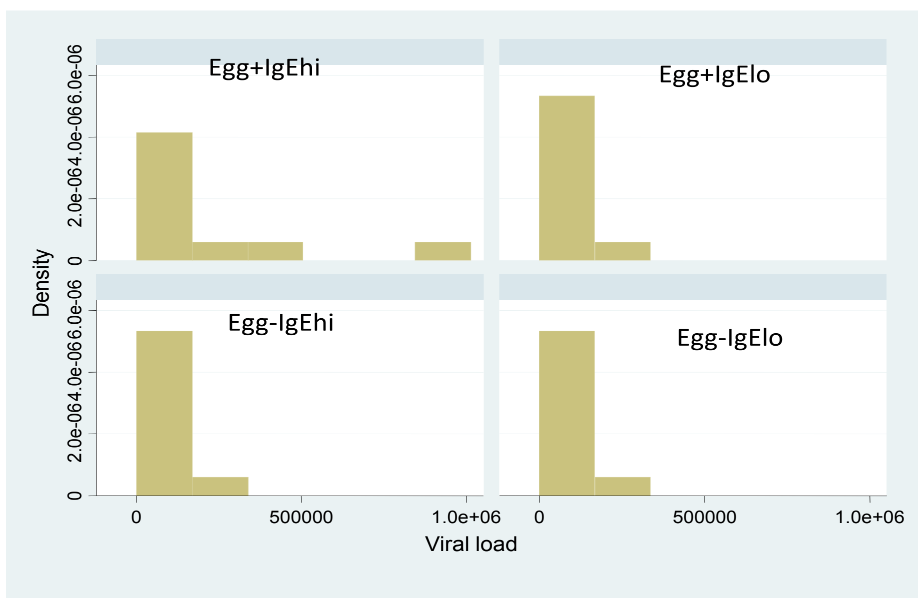


Figure 4.3 Histograms for distribution of viral load data in four subgroups of HIV positive subgroups with or without evidence of helminth infection. Ten individuals in each subgroup were randomly selected for analysis of expression of activation markers on T lymphocyte surfaces.

Table 4.3 Median percentage expression of surface activation markers on T lymphocyte subsets in HIV uninfected subgroups with or without helminth co-infection.

The subgroups were classified according to the presence or absence of faecal egg excretion and low or high serum Ascaris-specific IgE. Cryopreseved peripheral blood monocytes (PBMCs) were stained with fluorochrome- conjugated monoclonal antibodies to activation marker molecules and the proportions of T cell subsets expressing the particular markers quantitated by flow cytometry.

Group	Marker	CD4-CCR5	CD8 CCR5	CD4-CD25	CD4-HLA-DR	CD8-HLA-DR	CD3-HLA-DR	CD8CD38	CD4-D71	CD8 CD71	CD4
		p=0.36	p=0.09	p=0.61	p=0.24	p=0.09	p=0.23	p=0.12	p=0.59	p=0.11	**p=0.39**
Egg$^+$ IgE^{hi} (n=5)	**Median**	**4.3**	**20.1**	**42.6**	**11.8**	**13.8**	**14.4**	**50.3**	**3.5**	**2.8**	**0.55**
	Min	2.2	15.9	28.0	5.4	8.1	7.2	40.8	1.9	2.2	0.13
	Max	20.8	48.5	51.6	38.4	38.5	30.2	92.1	6.5	7.6	0.69
Egg$^+$ IgE^{lo} (n=7)	**Median**	**4.9**	**15.7**	**37.9**	**8.3**	**22.8**	**20.7**	**26.7**	**3.1**	**3.9**	**0.75**
	Min	1.96	11.0	13.6	4.1	8.1	8.7	18.0	1.5	2.2	0.43
	Max	6.9	21.7	62.1	12.6	36.2	21.8	67.0	7.0	6.3	1.18
Egg$^-$ IgE^{hi} (n=3)	**Median**	**2.2**	**11.3**	**29.9**	**5.9**	**9.5**	**8.9**	**55.4**	**5.0**	**5.2**	**0.81**
	Min	0.98	7.0	29.88	4.9	7.5	8.41	38.4	2.9	3.4	0.58
	Max	6.1	12.2	29.98	7.7	9.7	11.5	56.8	7.3	7.0	1.15
Egg$^-$ IgE^{lo} (n=7)	**Median**	**2.4**	**14.4**	**32.2**	**7.8**	**15.8**	**12.4**	**49.8**	**4.3**	**7.7**	**0.74**
	Min	1.2	8.3	21.3	4.5	11.7	9.3	16.8	3.1	4.0	0.4
	Max	22.1	47.7	49	15.5	21.1	21.3	85.3	5.9	9.8	.94

Values indicate median percentages for each subgroup. Subgroups showing trends are underlined.

Egg $^+$ IgE ^{hi}: faecal helminth eggs and high (>0.35KU/L) serum *Ascaris lumbricoides*-specific IgE;
Eggs$^+$ IgE^{lo} :faecal helminth eggs and low (<0.35KU/L) serum *Ascaris lumbricoides*-specific IgE;
Egg$^-$ IgE^{hi} no faecal helminth eggs and high serum *Ascaris lumbricoides*-specific IgE;
Egg$^-$ IgE^{lo} :no faecal helminth eggs and low serum *Ascaris lumbricoides*-specific IgE.

4.3.2 Expression of surface activation markers in HIV positive and negative subgroups

Flow cytometry analysis showed that expression of activation markers differed significantly between HIV positive and HIV negative groups (Figure 4.2) and also between subgroups within the HIV+ group according to helminth co-infection (Figures 4.4 and 4.5). Representative plots of some of the markers illustrating these differences are depicted in Figures 4.5- 4.6. Plots of HIV negative groups for comparison are shown in Appendices 2-3.

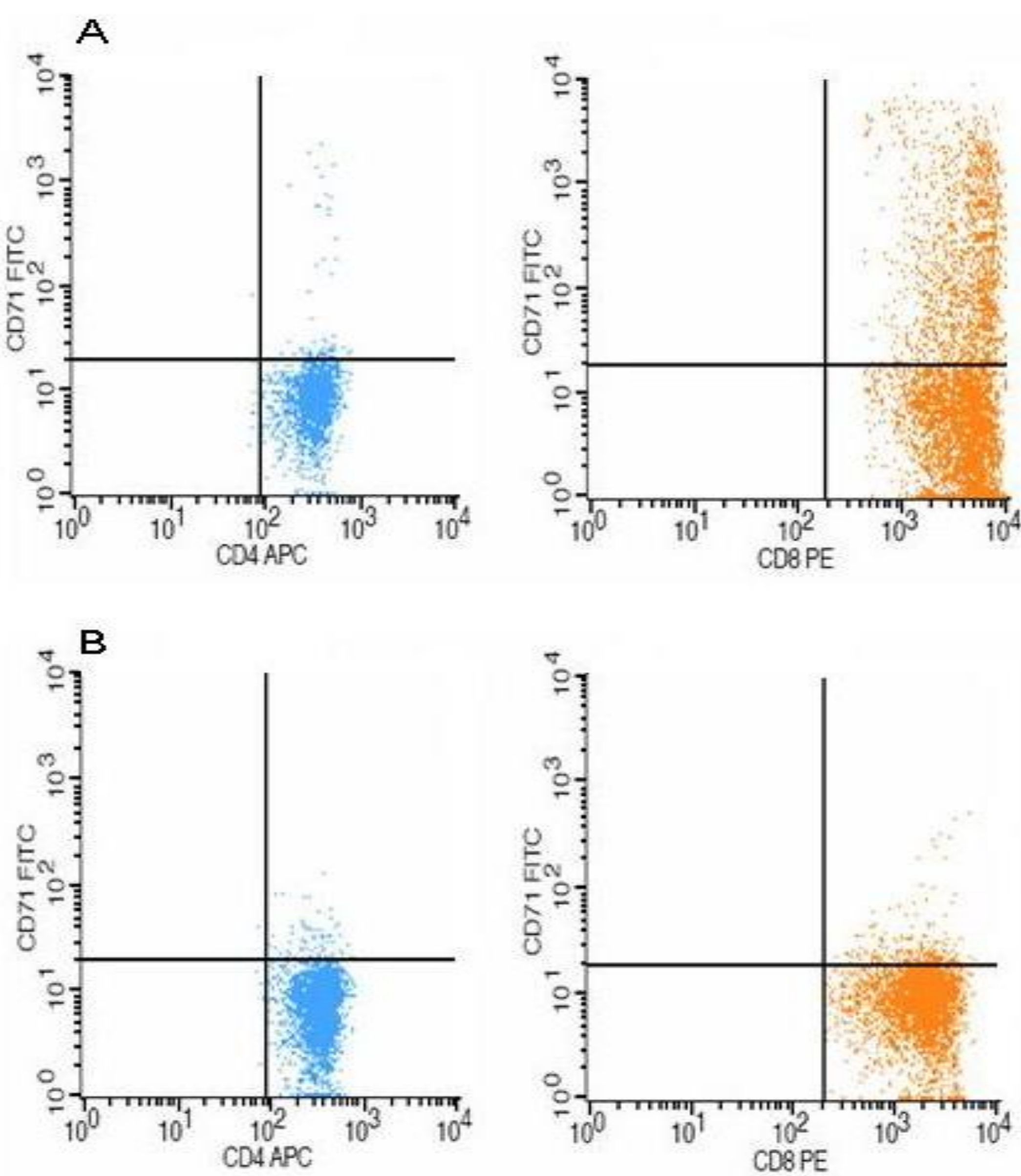
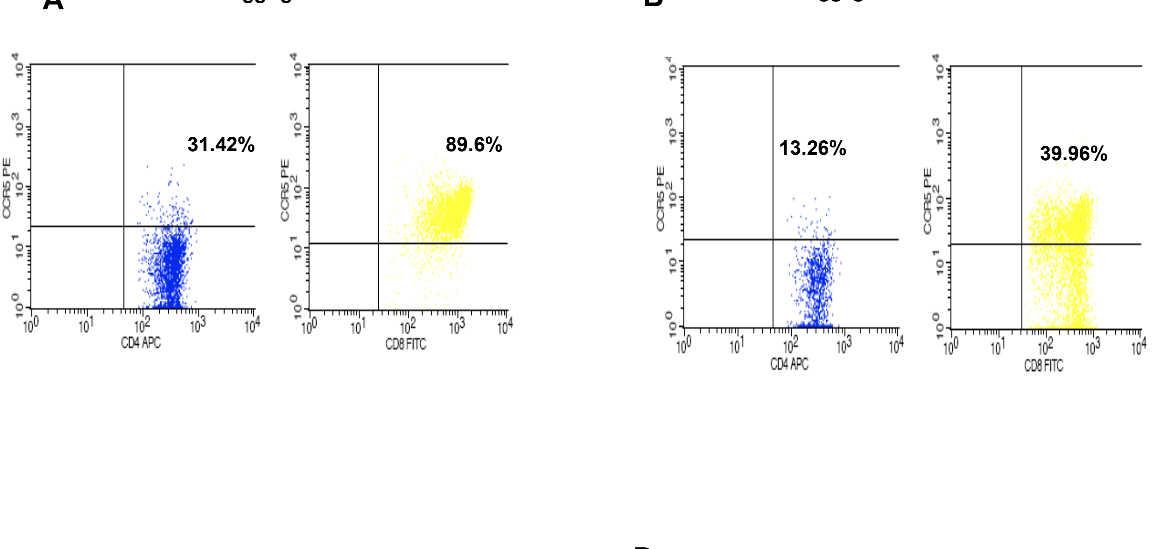


Figure 4.4 Expression of CD71 in CD4^{+} and CD8^{+} lymphocytes.

Ex *vivo* PBMCs were phenotyped by four colour flow cytometry and proportions of CD4^{+} and CD8^{+} cells expressing CD71 determined. Autofluorescence was controlled for by unstained cells from an HIV negative and helminth uninfected individual. Matched isotype control antibodies were used to set the cut-off for separating negative and positive populations. Each dot represents an event. Positive cells appear in the right upper quadrants. The figure represents results from individuals representative of the egg^{+}IgE^{hi} with HIV co-infection (A) and the egg^{-}IgE^{lo} HIV-1 singly-infected (B) groups. CD71 expression on CD8^{+} cells is markedly increased in an HIV+ individual with faecal helminth eggs and elevated serum *Ascaris* IgE (egg^{+}IgE^{hi}) (Panel A) compared to a HIV+ individual without evidence of intestinal parasite infection (egg^{-}IgE^{lo}) (Panel B).



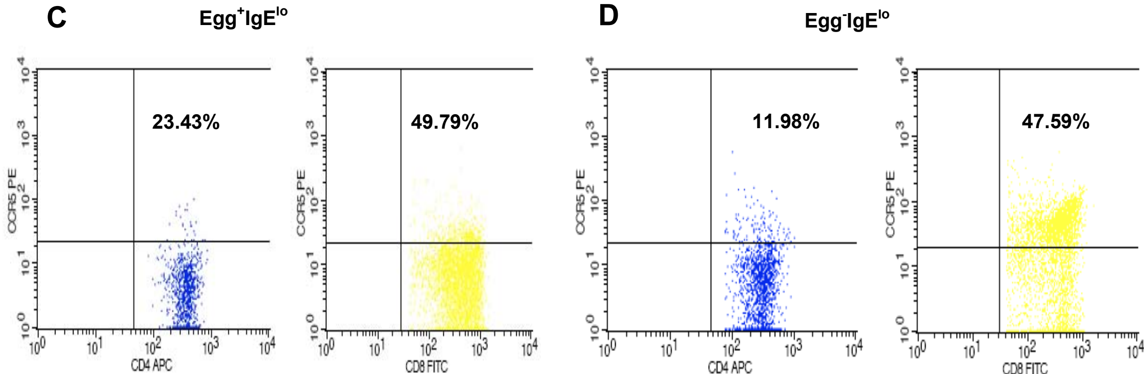


Figure 4.5 Representative plots: Expression of CCR5 by CD4+ and CD8+ cells in subgroups of HIV + individuals.

PBMCs were stained with flurochrome-tagged monoclonal antibodies to CD3, CD4, CD8 and CCR5 and analysed using flow cytometry to determine the percentages of lymphocytes expressing CCR5.. Positive cells appear in the right upper quadrant. The percentages represent the medians for each ~~cell~~ subgroup and each plot is a representative subject from each group. Expression of CCR5 on CD8^{+} cells was remarkably increased within the Egg^{+}IgE^{hi} subgroup (A).

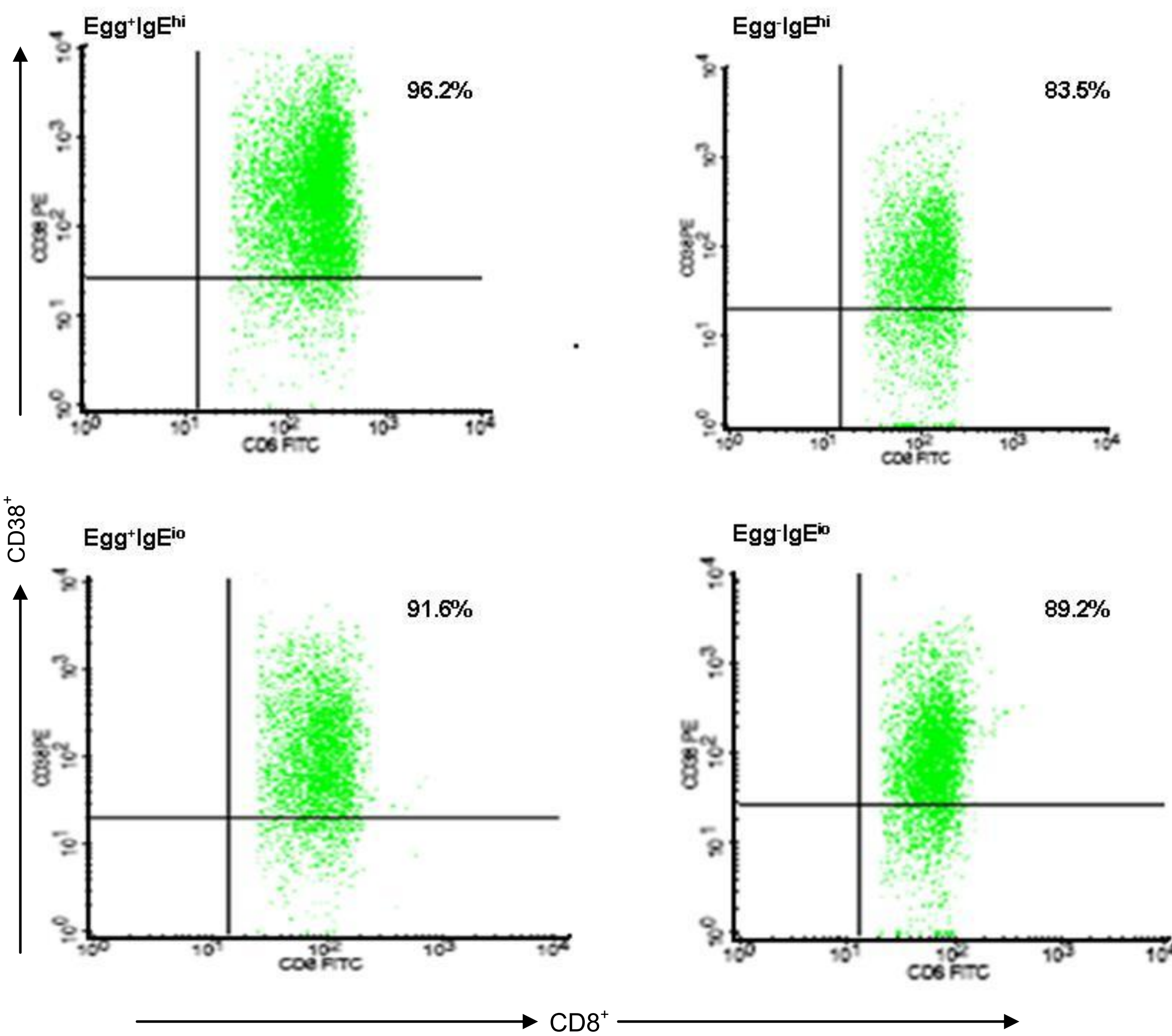


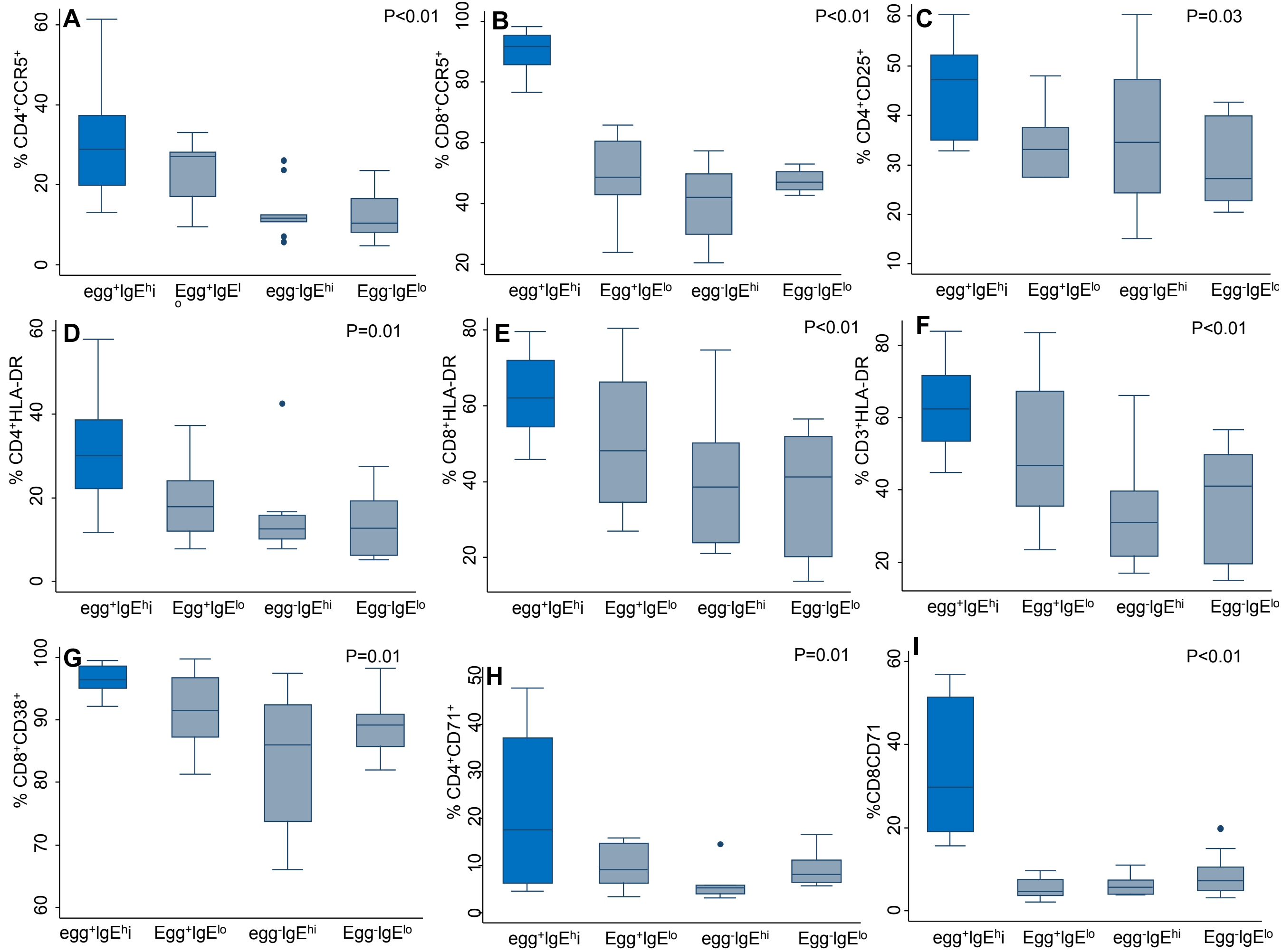
Figure 4.6 Representative plots: Expression of CD38 on CD8$^+$ cells in HIV$^+$ subgroups.
Ex *vivo* PBMCs were phenotyped by four colour flow cytometry and proportions of CD8$^+$ cells expressing CD38 determined. Autofluorescence was controlled for by unstained cells from an HIV negative and helminth uninfected individual. Matched isotype control antibodies were used to set the cut-off for separating negative and positive populations. Positive cells appear on the right upper quadrants. The percentages represent the median for each subgroup. Each dot plot is a representative plot of an individual in the respective group. The majority of individuals in the Egg$^+$IgE^{hi} group displayed almost universal expression of CD38 on CD8$^+$ cells.

4.3.3 Activation profile and interactions between subgroups

In order to determine interactions between subgroups with regards to HIV and helminth coinfections and their immunological implication, the median percentage of cells expressing the activation markers were compared by ANOVA (Kruskal Wallis) within subgroups of HIV^+ (Table 4.4 and Figure 4.7pages 149-150) and HIV^- groups (Figure 4.6 in pages 249-251).

Figure 4.7 Kruskal Wallis Box and whisker plots for lymphocyte surface expression of activation markers in HIV+ subgroups.

Ex vivo, cryopreserved peripheral blood mononuclear cells (PBMCs) were stained with fluorochrome-conjugated monoclonal antibodies to CCR5,HLA-DR, CD38 and CD71 and the proportions of T cells



expressing the particular markers quantitated by flow cytometry. Percentage median, 25-75 percentile, outlier and extreme values of these markers are shown for each subgroup and depicted along the y axis.Median percentage values of each subgroup was compared by Kruskal Wallis Anova for significant differences with bthe other subgroups. In all panels the groups and their sizes were: $Egg^{+}IgE^{hi}$ group (n = 10): helminth egg excretion and elevated *Ascaris lumbricoides* IgE; $Egg^{+}IgE^{lo}$ (n=10): helminth egg excretion and low Ascaris *lumbricoides* IgE; $Egg^{-}IgE^{hi}$ group (n=10): No helminth egg excretion and high *Ascaris lumbricoides* IgE; $Egg^{-}Ig^{lo}$ (n=10): No helminth egg excretion and low *Ascaris lumbricoides* IgE. Significantly different group /s shown in deep blue.

Table 4.4 Kruskal Wallis ANOVA, Median tests and multiple comparisons of Activation markers in HIV$^+$groups.

Lymphocyte expression of activation markers (CCR5, HLA-DR, CD25, CD38 and CD71) was measured by flow cytometry. Percentage medians of each activation marker were compared by Krushkal Wallis ANOVA for significant differences in each of four HIV$^+$ subgroups. P values are shown for all statistically significant interactions.

	(N)	*KRUSKAL-WALLIS ANOVA BY RANKS*	*MULTIPLE COMPARISONS 2-TAILED P VALUES*	*GROUPS WITH SIGNIFICANT INTERACTIONS*
CD4CCR5	**39**	**0.01**	**0.01**	**1 & 3**
			<0.01	**1 & 4**
			0.05	**2 & 4**
CD8CCR5	**39**	**<0.01**	**0.01**	**1&2**
			<0.01	**1&3**
			<0.01	**1&4**
CD4CD25	**40**	**0.03**	**0.04**	**1& 4**
CD4HLADR	**40**	**0.01**	**0.01**	**1 & 3**
			0.01	**1 & 4**
CD8HLADR	**40**	**0.01**	**0.04**	**1 & 3**
			0.01	**1 & 4**
CD3HLADR	**40**	**<0.01**	**0.01**	**1 & 3**
			0.02	**1 & 4**
CD8CD38	**40**	**0.01**	**0.01**	**1 & 3**
			0.03	**1 & 4**
CD4CD71	**40**	**0.01**	**0.01**	**1& 3**
CD8CD71	**40**	**<0.01**	**<0.01**	**1& 2**
			<0.01	**1 & 3**
			<0.01	**1& 4**

Group 1 – Egg$^+$IgE^{hi} ; Group 2- Egg$^+$IgE^{lo} Group 3- Egg$^-$IgE^{hi} Group 4- Egg$^-$IgE^{lo}
IgE^{hi}-high serum Ascaris specific IgE; IgE^{lo} – low serum *Ascaris lumbricoides*-specific IgE.

4.3.3.1 The egg$^+$/IgE^{hi} infection status is associated with significant increased expression of all activation markers in the HIV positive groups

The HIV$^+$, egg$^+$IgE^{hi} and egg$^-$IgE^{lo} subgroups:

When the HIV and helminth co-infected, egg$^+$IgE^{hi} subgroup was compared to the HIV-singly infected egg$^-$IgE^{lo} subgroup, both have similar median CD4+ counts, which were slightly higher (0.268 cells/ml) in the former compared to 0.22 cells /ml in the egg$^-$IgE^{lo} subgroup.The median viral load was significantly higher in the dually infected egg$^+$IgE^{hi} group (Table 4.2 page 142).It is noted that the viral loads could not be matched before random selection of the subgroups.This group was also found to express statistically-significantly higher levels of all activation markers (Table 4.2) than the singly-HIV, helminth non-infected (egg$^-$IgE^{lo}) subgroup and all the other subgroups in the HIV positive group, both of which had higher and similar median CD4+ counts (Table 4.2).

Expression of all measured activation markers was markedly increased in the egg$^+$/IgE^{hi} (typical helminth infection) group. All these differences were statistically highly significant ($p \leq 0.01$; and for CD4$^+$CD25$^+$ $p=0.03$) Figure 4.7 Panels A-I (page 149) and Table 4.2,). In this group, median CD3$^+$, CD4$^+$ and CD8$^+$ expression of HLA-DR, were higher than in all the other three subgroups (Table 4.2 and Figure 4.7 page 1497 panels D-F, $p \leq 0.01$). In addition, median expression of CCR5 by CD8$^+$ cells exceeded 90.0% while in CD4$^+$ cells median CCR5 expression was almost doubled in this egg$^+$/IgE^{hi} group compared to the egg$^-$/IgE^{hi} and the egg$^-$/IgE^{lo} groups (Table 4.2). Furthermore, the early activation marker-CD71 was 2-3 fold higher ($p<0.001$) than in the other three groups in both CD4$^+$ and CD8$^+$ compartments (Table 4.2 & Table 4.4 page 150; Figure 4.75 page 149 Panels H & I). In most instances these differences in CD71 expression on CD8$^+$ cells were distinct (Figure 4.4 page 145). Finally almost all the CD8$^+$ cells were CD38$^+$ in this group (Fig 4. 6 page 147 and Table 4.2 page 142) and this difference was also statistically highly significant ($p=0.01$).

The highest levels of percentages of lymphocytes expressing all activation markers by the egg$^+$IgE$^+$ group is remarkable in view of the fact that in the preceeding chapter (Section 3.2.3.1), this egg$^+$IgE^{hi} group was shown to have a tendency towards a generalized reduction of lymphocyte populations.

Comparisons between the $egg^{+}IgE^{hi}$ and the $egg^{-}IgE^{lo}$ groups show that median expression of all activation markers was almost two-fold higher in the former group (Table 4.2 page 142). These results show a significant difference between the $egg^{+}IgE^{hi}$ helminth/HIV co-infected group and the HIV-singly infected ($egg^{-}IgE^{lo}$) group with regards to the level of immune activation.

HIV negative groups

Among the HIV^{-} negative subgroups, the $egg^{+}IgE^{hi}$ subgroup had lower median CD4+ counts compared to the four subgroups. No dramatic increases in activation markers were observed among these individuals and no statistically significant differences were observed in any of the variables analysed (Table 4.3 page 144).

There was only apparent slight increase (nearly two-fold) in median $CD4^{+}$ $CCR5^{+}$ in the $egg^{+}IgE^{hi}$ and $egg^{+}IgE^{lo}$ participants compared to the $egg^{-}IgE^{hi}$ and the $egg^{-}IgE^{lo}$ subgroups. A trend towards higher $CD8^{+}$ HLA-DR (p=0,09) was observed between the $egg^{+}IgE^{lo}$ and the $egg^{-}IgE^{hi}$. Likewise, expression of CCR5 on $CD8^{+}$ cells displayed a trend towards higher levels between the $egg^{+}IgE^{hi}$ and $egg^{-}IgE^{hi}$ subgroups (Table 4.3).

4.3.3.2 Stool egg positivity is associated with increased CD4 $^{+}CCR5^{+}$ and $CD8^{+}$ $CD38^{+}$

The results show that the stool egg positive subgroups ($egg^{+}IgE^{hi}$ and $egg^{+}IgE^{lo}$) have more than two-fold median CCR5 expression by $CD4^{+}$ cells, compared to the stool egg negative subgroups ($egg^{-}IgE^{hi}$ and $egg^{-}IgE^{lo}$) in the HIV^{+} group (Table 4.2 page 142). These differences were either highly statistically significant (p<0.01 or nearly significant (p=0.05) - Table 4.4 page 142. Furthermore, in both stool egg positive subgroups, median expression of CD38 by CD8+ lymphocytes exceeded 90% and this was statistically significant (Table 4.2 and Figure 4.7 page 149i52 Panel G).

HIV negative groups

Among the HIV uninfected individuals, both stool egg positive groups ($egg^{+}IgE^{hi}$ and $egg^{+}IgE^{lo}$) had two-fold higher levels of $CD4^{+}$ $CCR5^{+}$ compared to the stool egg negative subgroups ($egg^{-}IgE^{hi}$ and $egg^{-}IgE^{lo}$) but this difference was not significant (p=0,36). A trend for higher $CD8^{+}$ CCR5 (p=0, 09) was also observed in both these subgroups with the egg positive status (Figure 4.8 in Appendix 4 page 258.)

4.3.4 Correlations between activation markers and surrogate markers of HIV progression.

Indicators of HIV disease progression ($CD4^+$ counts and viral load) were correlated with all measured activation markers (Appendix 5, Table 4,5B). This was aimed at evaluating any association between the level of activation and HIV disease progression in the context of HIV/helminth coinfection. Analysis of all the correlations using the Spearman rank order test (Appendix 5) showed that no significant interactions were observed between CD8CCR5 and both disease markers, while CD4CCR5 showed a strong negative correlation in the two groups with high IgE(r =-0.75, p=0.01 andr=-0,72, p=0.03).No correlations between viral load and CD4 CCR5 were observed except for the egg^+IgE^{hi} subgroup with a strong positive correlation r=0.72, p=0.01.CD4 CD71 correlated negatively with CD4 counts in the egg^+IgE^{lo} group (r=--0.81(p=0.02).

Further analysis was done on the classic marker of activation (HLA-DR) and on CD8CD38 because of its association with HIV disease prognosis. Because correlation values for CD4+HLA-DR and CD8+HLA-DR were similar to CD3+ HLA-DR, (Appendix 5) the CD3+HLADR correlations were used as a representative of all T lymphocyets interactions reported together with the correlations between viral load and CD4+ counts and the prognosis marker(CD8CD38) in Table 4.5. (It is noted that for practical reasons by experimental design, CD38 expression could only be assessed on CD8+ cells, and this was sufficient for purposes of evaluation of activation)

4.3.4.1 Correlation between $CD3^+$ HLA-DR and $CD4^+$ counts

Analysis of all forty HIV positive individuals as one group, using the Spearman rank order test showed a significant negative correlation between HLA-DR expression on lymphocytes and total $CD4^+$ counts. This inverse correlation was significant (r=-0.53, p=0.04) (and strong in the egg^+IgE^{hi} group (Table 4.5).

4.3.4.2 Correlation between CD8$^+$CD38$^+$ and CD4$^+$ counts

When all the HIV positive participants were analysed in one group, a significant inverse correlation was observed between CD8$^+$CD38$^+$ and CD4$^+$ counts (Figure 4.8). Further analysis of subgroups showed that this correlation was strongly significant in the two groups with high *Ascaris* IgE (r=-0.69, p=0.03 and r = -0.72, p=0.02). Both groups with low IgE had weak correlations between CD8$^+$CD38$^+$ and CD4$^+$ counts that was weakest in the egg$^-$ IgE^{lo} group (Figure 4.8 and Table 4.5).

Scatterplots for Activation vs CD4+ counts

All the HIV+ group n=40

The HIV+ egg+IgEhi subgroup n=10

The HIV+egg-IgEhi subgroup n=10

The HIV+egg-IgElo subgroup n=10

Figure 4.8 Scatterplots for activation versus CD4+ counts in HIV+ groups. The distribution of CD4+ conts in relation to CD8CD38 expression in HIV+ groups with or without helminth coinfection.

4.3.4.3 Correlation between CD3$^+$ HLA-DR and viral load

Comparison of CD3$^+$HLA DR and viral load showed a significant positive correlation in the entire group of HIV positive individuals. However this correlation was only significant

in the egg$^-$ IgE^{hi} individuals when subgroups were analysed separately. In the rest of the subgroups the correlation was not significant (Table 4.5).

4.3.4.4 Correlation between CD8$^+$CD38$^+$ and viral load

A strong and significant positive correlation was found between CD8$^+$CD38$^+$ and viral load in all forty HIV positive individuals analysed as one group. The positive correlation was strongest in the egg$^-$ IgE^{hi} group followed by the egg$^+$ IgE^{lo} group. No correlation was found in the egg$^+$ IgE^{hi} and egg$^-$ IgE^{lo} groups (Table 4.5).

Table 4.5 Correlations between activation markers and HIV disease progression indicators ($CD4^+$ counts plus viral load).

Spearman rank analysis of correlations between $CD4^+$ and $CD3^+$ HLA-DR, $CD8^+CD38^+$ and between viral loads was undertaken in HIV^+ groups.

Parameter	Group	N	$CD3^+HLA\text{-}DR^+$		$CD8^+CD38^+$	
			Spearman r	**P value**	**Spearman r**	**P value**
$CD4^+$	All	40	-0.43	0.01	-0.44	0.01
	1(*Egg^+ IgE^{hi})	10	-0.7	0.03	-0.69	0.03
	2 (Egg^+ IgE^{lo})	10	-0.54	0.11	-0.43	0.21
	3 (Egg^- IgE^{hi})	10	-0.59	0.07	-0.72	0.02
	4 (Egg^- IgE^{lo})	10	-0.56	0.09	-0.17	0.63
Viral Load	All	40	0.49	<0.01	0.68	<0.01
	1(*Egg^+ IgE^{hi})	10	0.49	0.14	0.07	0.85
	2 (Egg^+ IgE^{lo})	10	0.07	0.86	0.69	0.03
	3 (Egg^- IgE^{hi})	10	0.65	0.04	0.72	0.02
	4 (Egg^- IgE^{lo})	10	0.01	0.9	0.29	0.41

* Eggs= faecal helminth eggs; high IgE = high serum *Ascaris lumbricoides*-specific IgE. All =all subgroups analysed as one group.

4.3.5 Comparison of HIV uninfected, helminth negative results with other studies

In order to determine from the current study whether HIV negative individuals residing in local, resource limited settings (data from Table 4.3) present with an immune activated profile, a comparison with other published flow-cytometry-based studies is shown in Table 4.6 page 151. Comparison of the values obtained from presumably healthy participants in the present study with other population groups revealed similarly elevated

CD8CD38 in healthy Chinese and Ethiopians as well as the other healthy, HIV negative South African adults (Table 4.6). CD4 and CD8 HLA-DR values in the present study were comparable to the Ethiopian median values, which were all higher than those obtained from the Israeli controls. CCR5 was lower in the present study when compared to the results from the Chinese and the other South African studies. Although the numbers in the present study are smaller, the results presumptively suggest relatively high levels of expression of activation markers among HIV negative, helminth uninfected individuals residing in resource-poor setting in South Africa.

Table 4.6 Comparison of activation marker levels in different ethnic groups. Comparison of immune activation data (percentage expression) from HIV uninfected subjects of different ethnic groups as published in other studies. References are listed in the table legend. Values in brackets refer to reference 3.

Marker	***Population Studied***					
	Ethiopians [2]**N=63** [3]**(N=26)**	**Israelis** [2]**N=34** [3]**(N=20)**	**†Old migrant Ethiopians** [2]**N=18** [3] **(N=22)**	**Chinese** [1]**N=13**	**Johannesburg (South Africa)** [4] **N=14** [5] **N=18**	**Khayelitsha (South Africa) N=7**
Data value	Mean	Mean	Mean	*Geo.mean	Median	Median
CD4 CCR5	(26,8)	13,1	25,6	21,1	[4]23,0	2,4
CD8 CCR5	Nd	Nd	Nd	49,6	[4]44,5	14,4
CD4 HLA-DR	6,5 (12,5)	2,9 (4,9)	3,5 (5,4)	15,1	Nd	7,8
CD8HLA-DR	16,2	6,2	9,8	28,9	Nd	15,8
CD8 CD38	56,4	34,5	42,5	51,8	[4]50,7 [5]48,0	49,8

1. Shang *et al*., 2005; 2. Kalinkovinch *et al*., 1998; 3. Kalinkovinch *et al.,* 2001; 4. Shalekoff *et al*., 2001; 5. Shalekoff *et al*., 2004; Khayelitsha-Data from Table 4.3 in the present study. †Old migrant Ethiopians refers to Ethiopians who had immigrated to Israel and treated for helminth infections; Nd- Not done;* Geo. Mean –geometric mean.

4.4 Discussion

In this section, individuals in the four subgroups of the HIV^{+} group were randomly selected into each subgroup before viral load and $CD4^{+}$ counts were available, hence the subgroups could not be matched for these variables. Subsequent analysis of these subgroups showed that the $egg^{+}IgE^{hi}$ had the highest median viral load compared to all thre subgroups while the median viral load for the $egg^{-}IgE^{lo}$ was lowest. These two groups had similar median CD4+ counts (slightly higher, 0.27 counts/ml in the $egg^{+}IgE^{hi}$ and lower, 0.22 counts/ml in the $egg^{-}IgE^{hi}$ subgroup). This finding suggests that the two groups had similar levels of immunocompromise but were virologically very different. The results of this section have shown a significant association between immune activation and $egg^{+}IgE^{hi}$ status in HIV co-infection. Furthermore, immune activation inversely correlated with markers of HIV disease progression particularly as determined by $CD4^{+}$ counts in the co-infected individuals.

A prominent finding was the highly significant association between immune activation (as determined by surface T cell expression of HLA-DR, CD38, CCR5 and CD71) and $egg^{+}IgE^{hi}$ status where all these markers were significantly elevated in the $egg^{+}IgE^{hi}$ and HIV co-infected group. Notably this increased expression of activation markers in the $egg^{+}IgE^{hi}$ group was more than double compared to the levels in the HIV singly-infected (without evidence of helminth infection) $egg^{-}IgE^{lo}$ group. No significant increase in immune activation was noted in the HIV-singly infected $egg^{-}IgE^{lo}$ group. The association between immune activation and higher viral load found only in the dually infected $egg^{+}IgE^{hi}$ group concurs with the suggested notion that co-infection with HIV and helminths would increase immune activation and thereby possibly accelerate HIV replication. This is supported by the finding that the HIV-singly infected $egg^{-}IgE^{lo}$ subgroup had lower viral load and no increase in immune activation marker expression. The fact that in both HIV+ single infection ($egg^{-}IgE^{lo}$) and helminth single infection (HIV-$egg^{+}IgE^{hi}$) there was no remarkable increase in immune activation strongly suggests that dual infection by helminths and HIV increases immune activation which in part or wholly drives virus replication. From our findings it is apparent that this effect is pronounced during a typical helminth ($egg^{+}IgE^{hi}$) infection.

In the HIV negative group, the helminth singly-infected $egg^{+}IgE^{hi}$ subgroup had lowest $CD4^{+}$ counts, just as the HIV singly infected ($egg^{-}IgE^{lo}$ subgroup) also had lowest CD4+ counts albeit lowest median viral load. In both instances immune activation was not increased, suggesting that other factors independent of immune activation or virus burden could contribute to lower CD4+ counts during infections. This also tallies with the suggestion that dual infection increases both immune activation and virus replication.

Other important findings that support the study hypotheses were observed: firstly increased expression of CCR5 in $CD4^{+}$ cells was associated with stool egg excretion irrespective of serum *Ascaris* IgE levels. This was reflected by significantly higher levels of $CD4^{+}CCR5^{+}$ in the two stool egg positive subgroups (the $egg^{+}IgE^{hi}$ and $egg^{+}IgE^{lo}$ subgroups) compared to the stool egg negative subgroups (the $egg^{-}IgE^{hi}$ and the $egg^{-}IgE^{lo}$ groups). Both CD4 and CCR5 are known to be critical mediators of HIV infection as viral receptors, thus increased levels of both receptors could increase susceptibility to HIV infection and enhance the virus replication cycle by increasing the rate of infection of new cells bearing these receptors. Under this premise, the findings are in support of the study hypothesis that helminth infection increases HIV replication.

CCR5 expression is regulated by cytokines; in particular IL-10 has been shown to upregulate its expression (Bleul *et al*., 1997; Sozzani *et al*., 1998) and is predominantly expressed on memory cells (Bleul *et al*., 1997). Helminths are documented to increase both the memory ($CD45RO^{+}$) cell pool (Kalinkovinch *et al*., 1998) and IL-10 production (Borkow and Bentwich, 2004). It is therefore expected that under conditions of continuous helminth exposure, CCR5 would be elevated. Indeed in this study, $egg^{+}IgE^{hi}$ helminth infection was associated with elevated CCR5 levels in both HIV^{+} and HIV^{-} groups although this was not statistically significant in the HIV^{-} group though a strong trend was observed in the $CD8^{+}$ subset (Tables 4.2 - 4.4). High levels of CCR5 were especially evident in $CD8^{+}$cells and more prominently in HIV^{+} individuals.

The association between stool egg excretion and elevated $CD4^{+}CCR5^{+}$ found in this study is consistent with the findings reported by Secor *et al.* (2003) where patients with active schistosomiasis were found to express higher surface densities of both CCR5 and CXCR4 than cured schistosomiasis patients. Similarly, co-infection with TB and HIV was shown to be associated with elevated CCR5 in both $CD4^{+}$ and $CD8^{+}$ lymphocytes

(Shalekoff *et al.*, 2001). In the present study, the HIV and helminth coinfected group ($egg^{+}IgE^{hi}$) had the highest levels of $CD4^{+}$ and $CD8^{+}$ CCR5 expression compared to all the subgroups. Expression of chemokine receptors (including CCR5) and their respective ligands facilitates migration of immune cells to sites of antigen presentation (Janeway *et al.*, 2001). CCR5 upregulation during recurrent antigenic challenge (as would occur during chronic helminth and HIV infections) is expected and the findings of the present section concur with this concept.

Secondly, in the HIV^{+} group, the median expression of CD38 on $CD8^{+}$ cells was 97% in the $egg^{+}gE^{hi}$ group, and this was statistically highly significant (p= 0.01) when compared to the $egg^{-}IgE^{hi}$ subgroup, and also significant (p<0.03) compared to the $egg^{-}IgE^{lo}$ subgroup (Table 4.4 page 150). The $egg^{+}gE^{lo}$ group also had 92.0% median $CD8^{+}CD38^{+}$, thus CD38, a poor prognosis marker for HIV disease (Liu *et al.*, 1997; Shang *et al.*, 2005) was elevated in both subgroups with stool eggs. This increase in CD38 expression among stool egg positive subgroups could suggest more advanced HIV disease in these individuals. In the context of the study hypothesis, these results suggest that helminth infection that is associated with stool egg excretion in the presence of HIV increases the level of immune activation and since CD38 elevation is associated with poor prognosis, these results imply that HIV progression may be particularly enhanced by a typical fecund (egg^{+}) intestinal helminth infection. However it should be noted that all HIV infected subgroups had more than 80.0% median $CD8^{+}CD38^{+}$ in this study (Table 4.2 page 142).

Thirdly, analysis of correlations also showed significant associations between the level of immune activation and indicators of HIV disease progression in HIV/helminth co-infected individuals. These were more clearly evident when disease progression was assessed by absolute $CD4^{+}$ counts. Expression of the characteristic marker of activation (HLA-DR) on lymphocytes negatively correlated with $CD4^{+}$ counts (Spearman r = -48) and this was more significant in the $egg^{+}IgE^{hi}$ group (Spearman r =-0.70, p=0.03). This finding strongly suggests that $egg^{+}IgE^{hi}$ helminth infection induces immune activation which has a negative influence on the host’s ability to respond to infections including HIV because $CD4^{+}$ cells are an integral part of a competent HIV immune response.

In addition, CD38 expression, the prognostic marker of HIV progression correlated negatively with $CD4^+$ counts (Spearman r = -0.44) and this negative relationship was more significant in both groups with high Ascaris-specific IgE (Spearman r = -0.69 and -0.72 respectively-Table 4.5 page 155). High IgE is associated with a Th_2 biased response to helminths. This negative association between $CD8^+CD38^+$ and $CD4^+$ counts in the Th_2 biased subgroups further suggests a negative influence of helminths on HIV disease progression. Indeed, Liu *et al*. (1997) showed that upregulation of the CD38 antigen is associated with $CD4^+$ T cell depletion and is a stronger indicator of progression to AIDS. Our results agree with their finding, and they are in support of the hypotheses being tested in the present study.

In parallel analyses of correlations, viral load was also used as an indicator of advanced HIV disease. A positive correlation was observed between viral load and $CD3^+$ HLA-DR, and also between viral load and $CD8^+$CD38 expression among all the HIV infected individuals. However, these relationships were not clearly interpretable amongst the subgroups in the context of co-infection. Interpretation of these observations suggests that generally the higher level of immune activation correlated with higher virus burden in the entire HIV infected group.

Overall results of correlations between immune activation and $CD4^+$ counts and viral loads in the present study concur with those reported by Leng *et al*. (2001), where they compared the correlation between immune activation and $CD4^+$ counts and viral load. The authors found that $CD4^+$ counts correlated better with activation than did viral load (Leng *et al*., 2001). They found a significant positive correlation between viral load and immune activation but it was not as strong as the correlation between immune activation and $CD4^+$ T cells. Furthermore, Sousa *et al.* (2002) demonstrated a direct causal relationship between immune activation and $CD4^+$ depletion, and that viral load is indirectly related to immune activation. These results also corroborate the hypothesis suggesting that immune activation drives $CD4^+$ T cell depletion (Sousa *et al*., 2002; Grossman *et al*., 2006) and is indirectly related to virus burden (Sousa *et al*., 2002).

Taken together, these findings provide suggestive evidence that immune activation induced by helminths may increase virus replication as indicated by increased virus load in the dually-infected subgroup. This is in agreement with the key proposition of the

study hypothesis: helminth infection induces immune activation which in turn enhances HIV disease advancement. The finding that the HIV negative, $egg^{+}IgE^{hi}$ subgroup had lowest median CD4+ counts may suggest an association between helminthiasis and lower CD4+ counts, which were also found in the HIV+ $egg^{+}IgE^{hi}$ group, (although the HIV+ $egg^{-}IgE^{lo}$group also had low median CD4+ count).

The classic immune response to worm antigens is characterized by strong Th_2 biasing that is accompanied by downregulatory circuits resulting from increased T_{reg} activity, alternatively-activated macrophages and dendritic cells that induce Th_2 cell differentiation (Rodriguez-Sosa *et al.*, 2002; Maizels and Yazdanbakhsh, 2003; Maizels *et al.*, 2004; Diaz and Allen, 2007) and selective depletion of effector cells (Borkow and Bentwich, 2004). Literature review suggests that these mechanisms of immune dysregulation are evolutionary parasite strategies of downmodulating the inflammatory host responses directed against them and also minimizing host tissue damage (Maizels and Yazdanbakhsh, 2003). This parasite response then effectively attenuates HIV immune responses through Th_1-Th_2 counterbalancing (Diaz and Allen, 2007); selective depletion of the effector memory pool, and generalized anergy induced by T_{regs} (Maizels and Yazdanbakhsh, 2003; Borkow and Bentwich, 2004). Owing to the chronic nature of helminthiasis, activation of such responses is also persistent, thereby exerting a long lasting negative influence on anti-HIV immunity during co-infection. Such biased, chronic activation also provides a favourable mileu for virus replication through activation of pro-transcription factors and upregulation of HIV receptors. (Lawn *et al.*, 2001) within an anergic immune environment (Borkow and Bentwich, 2004).

An analysis of the activation profile in the absence of HIV infection was also undertaken to determine if helminth infection alone results in increased expression of activation markers. No dramatic increase in all activation markers was seen in theHIV negativt groups. However, in the HIV uninfected, egg^{+}/IgE^{hi} group a tendency towards increased expression of $CD8^{+}CCR5$, was observed. In addition, the egg^{+}/IgE^{hi} and the $egg^{+}IgE^{lo}$ groups had almost twofold levels of $CD4^{+}CCR5$ compared to the other subgroups. Conspicuously, $CD8^{+}CCR5^{+}$ and $CD4^{+}HLA\text{-}DR^{+}$ were highest relative to all the other HIV negative subgroups in the egg^{+}/IgE^{hi} group although these differences were not statistically significant. The small numbers in the subgroups of HIV uninfected individuals probably reduced the statistical power for this analysis.

Furthermore, it is reported that approximately 20.0% of $CD8^+$ are $CD38^+$ in healthy individuals (Kunkl, 1997). However, Kalinkovinch *et al.* (2001) reported a mean value of 34.0% among healthy Israelis, and 43.0-56.0% among healthy immigrant Ethiopians in Israel. Shang *et al* (2005) reported a mean level of 52.0% of $CD8^+CD38^+$ among 13 healthy Chinese participants. Our results (median 49.8% $CD8^+CD38^+$ in the HIV negative controls) are similar to both the Ethiopian and Chinese results. In two other South African studies, (Johannesburg, Gauteng Province) median CD38 expression on $CD8^+$ cells obtained from healthy adult African donors were 50,7% and 48.0% (Shalekoff *et al.*, 2001; 2004).

The percentage of median $CD8^+CD38^+$ expression in the populations cited above is higher than the 20.0% stipulated by Kunkl (1997), which is probably based on European/ Caucasian reference values. The relatively elevated $CD8^+CD38^+$ among HIV negative normal individuals reported previously in these, and the present study is consistent with the suggestion that generalised immune activation is present in populations residing in developing countries as a result of chronic exposure to infectious agents (Bentwich *et al.*, 1995; 1996; 1997; 1999; Clerici *et al.*, 2000; 2001). Considering the unsanitary conditions and poverty in Khayelitsha (as described in Chapter 2), it would be plausible that these individuals are constantly exposed to enteric helminths and other pathogens and thus chronically immune activated.

Finally, it is acknowledged that the other immunological hypothesis regarding the interaction between HIV and helminths suggests the possibility that HIV infection and progression could positively influence the pathogenesis of helminthiasis. Brown *et al.*(2006) have reviewed the bi-directional effects of both infections and highlighted several confounding in the studies that have addressed this concept. In the present study, there is suggestive evidence that the exposure to helminths probably preceded HIV infection in this cohort. More than 70% of the participants remembered previous epidsodes of helminth infection both when they were children and when they were adults. Approximately 60% recalled childhood worm infestation (Adams *et al.*, 2006). Under these circumstances and their living conditions, it is more likely that recurrent exposure to worms was a common event before HIV seroconversion, thus the immune

response to helminths existed prior to HIV exposure. Our approach therefore looked at the influence of helminths on HIV immune response.

4. 5 Conclusions

This work confirms that $egg^{+}IgE^{hi}$ helminth status during HIV co-infection results in a highly activated immune profile as reflected by markedly increased expression of all activation markers. This was associated with a higher median viral load in the typical heminth and HIV co-infected subgroup. Notably the poor prognosis marker, CD38 was highly expressed by $CD8^{+}$ cells in this subgroup and there was a strong, significant negative correlation between HLA-DR expression on lymphocytes and the absolute $CD4^{+}$ counts. The finding that almost all the $CD8^{+}$ cells were CD38 positive in this group and the negative correlation between increased immune activation and low $CD4^{+}$ counts further suggest that HIV progression could be accelerated in the individuals with the $egg^{+}IgE^{hi}$ profile of helminth infection.

Secondly, the $egg^{+}IgE^{hi}$ and the $egg^{+}IgE^{lo}$ subgroups (egg excretion positive status) and HIV co-infection presented a more activated profile compared to the stool egg negative subgroups, as shown by elevated CCR5 on $CD4^{+}$ cells as well as more than 90.0% median expression of CD38 on $CD38^{+}$ cells in both egg positive groups. These observations suggest that egg excretion increases immune activation and may increase susceptibility to, and accelerate HIV infection.

Thirdly, a strong negative correlation between CD38 expression and $CD4^{+}$ counts was found in both groups with high Ascaris IgE (the $egg^{+}IgE^{hi}$ and the $egg^{-}IgE^{hi}$ groups).

The consistent inverse correlation between immune activation and low $CD4^{+}$ cell numbers that was not found between viral load and immune activation could suggest that immune activation impacts more significantly on $CD4^{+}$ cell depletion than it does on viral load *per se* in HIV-helminth co-infections. It is noteworthy that a positive correlation between viral load and CD38 expression was found in the group of all the HIV positive individuals, confirming that CD38 expression is associated with advanced HIV disease. The results overall show a negative association between immune activation and

surrogate markers of HIV disease progression, and this was more evident in individuals with $egg^{+}IgE^{hi}$ worm infection and in Th_2- biased participants (high *Ascaris* IgE responders) which agrees with the hypothesis that helminth infection may contribute to HIV pathogenesis through exacerbation of immune activation and Th_2 predominance.

Furthermore, the sample of HIV negative individuals residing in Khayelitsha as represented by the HIV-uninfected and helminth negative group in this study also tended to present an activated immune profile. It is acknowledged however, that the HIV uninfected participant numbers was too small (N=7) to make conclusive inferences for the general population.

References.

Babusíková O, Ondrácková V, Prachar J, *et al.* 1999. Flow cytometric analysis of some activation/proliferation markers on human thymocytes. *Neoplasma* **46:** 349-55.

Bentwich Z, Kalinkovich A, WeismanZ. 1995. Immune activation is a dominant factor in the pathogenesis of African AIDS. *Immunology Today.***16 (4):** 187-191.

Bentwich Z, Weisman Z, Moroz C, Bar-Yehuda S, Kalinkovich A. 1996. Immune dysregulation in Ethiopian immigrants in Israel: relevance to helminth infections**?** *Clinical Experimental Immunology.* **103:** 239-243.

Bentwich Z, Weisman Z, Grossman Z, Galai N., Kalinkovich A. 1997. Pathogenesis of AIDS in Africa - lessons from the Ethiopian immigrants in Israel. *Immunologist* **5:** 21-26.

Bentwich Z, Kalinkovich A, Weisman, Z, *et al.* 1999. Can eradication of helminthic infections change the face of AIDS and tuberculosis? Immunology Today.**20 (11):** 485-487.

Bleul CC, Wu L, Hoxie JA, *et al.* 1997.The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proceedings of the National Academy of Science USA.* **94:**1925-1930**.**

Borkow G, Bentwich Z. 2004. Chronic immune activation associated with chronic helminthic and Human Immunodeficency Virus infections: Role of hyporesponsiveness and anergy. *Clinical Microbiology Reviews.* **17(4):** 1012-1030.

Brown M, Mawa PA, Kaleebu P, Elliott M. 2006. Helminths and HIV infection: epidemiological observations on immunological hypotheses. *Parasite Immunology.* **28(11):** 613–623.

Clerici M, Stefano B, Matthew L. *et al.* 2000. Immune activation in Africa is enviromentally-driven and is associated with upregulation of CCR5.*AIDS* **14 (14):** 2083-2092.

Clerici M, Declich S, Rizzardini G. 2001. African enigma: key player in Human Immunodeficiency Virus pathogenesis in developing countries? *Clinical and Diagnostic Laboratory Immunology.* **8(5):**864-866.

Diaz A, Allen JE. 2007. Mapping immune response profiles: The emerging scenario from helminth immunology. *European Journal of Immunology.* **37:**1-8.

Giorgi JV, Hultiri LE, McKeating JA, *et al.*1999. Shorter survival in advanced human immunodeficiency virus type 1infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus coreceptor usage. *The Journal of Infectious Diseases.***179 (4):**859-870.

Grossman Z, Meier-Schellersheim M, Sousa AE, *et al.* 2002. CD4 T cell depletion in HIV infection: are we closer to understanding the cause? *Nature Medicine.* **8:** 319.

Hazernberg MD, Otto SA, van Benthem BH, *et al.* 2003. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* **17:**1881-1888.

Iqbal SM, Ball TB, Kimani J, et *al.* 2005. Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1- resistant Kenyan commercial sex workers. *Journal of Infectious Diseases.* **192:**728-737.

Janeway CA, Travers P, Walport M, Shlomchik M. 2001**.** *In:* Schanck D, Austin P, Lawrence E, *et al.* eds. *T-cell mediated immunity***.** *Immunobiology . The immune system in health and disease. 5th ed. Chapters 1-14.* Garland Publishing. Taylor and Francis group.

Kalinkovinch A, Borkow G, Weisman Z**,** *et al.* 2001. Increased CCR5 and CXCR4 expression in Ethiopians living in Israel: environmental and constitutive factors. *Clinical Immunology.* **100:**107-117.

Kalinkovich A, Weisman Z, Greenberg Z, *et al.* 1998. Decreased CD4 and increased CD8 counts with T cell activation is associated with chronic helminth infection. *Clinical Experimental. Immunology.* **114 :** 414-421.

Kalinkovinch A, Weisman Z, Leng Q, *et al.* 1999. Increased CCR5 expression with decreased beta chemokine secretion in Ethiopians/: relevance to AIDS in Africa.*Journal of Human Virology.* **2 (5):** 283-289.

Kalinkovich A, Borkow G, Weisman Z**,** *et al.* 2001. Increased CCR5 and CXCR4 expression in Ethiopians living in Israel: environmental and constitutive factors. *Clinical Immunology.* **100:**107-117.

Kassu A, Tsegaye A, Wolday D *et al.* 2003. Role of incidental and/or cured intestinal parasitic infections on profile of $CD4^+$ and $CD8^+$ T cell subsets and activation status in HIV-1 infected and uninfected adult Ethiopians. *Clinical Experimental Immunology.* **132:**113-119.

Kunkl A. 1997.Flow cytometric immunophenotyping of HIV infected participants and quality control**.** In: *The Purdue Cytometry* CD-ROM Volume 3, J. Parker, C. Stewart, Guest Eds., J.Paul Robinson, Publisher. Purdue University, West Lafayette, 1997, ISBN 1-890473-02-2

Lawn SD, Butera S, Folks TM. 2001. The contribution of immune activation to the pathogenesis and transmission of Human Immunodeficiency Virus type1 infection. *Clinical Microbiology Reviews.* **14(4):** 753-777.

Leng Q, Borkow G, Weisman Z,*et al.* 2001. Immune activation correlates better than HIV plasma viral load with CD4 Tcell decline during HIV infection. *Journal of Acquired Immune Deficiency Syndromes.* **27:**389-397.

Liu Z, Cumberland WG, Hultin LE, *et al.* 1997. Elevated CD38 antigen on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than the CD4+ cell count, soluble immune activation

markers or combinations of HLA-DR and CD38 expression. *Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology.* **16:**83-97**.**

Mahalingam M, Peakman M, Davies ET, *et al*., 1993. T cell activation and disease severity in HIV infection. *Clinical Experimental Immunology.* **93:**337-343.

Maizels RM, Balic A, Gomez-Escobar N, *et al*. 2004. Helminth parasites- masters of regulation*. Immunological Reviews***. 201.** 89-116.

Maizels R, Yazdanbakhsh M. 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature Reviews Immunology***. 3:**733-744.

McKee AS, Pearce EJ. 2004. $CD25^{+}CD4^{+}$ cells contribute to Th2 polarisation during infection by suppressing Th1 response development. *The Journal of Immunology***.173:** 1224-1231.

Ostrowski MAS, Justement SJ, Cantazaro A, *et al*. 1998. Expression of chemokine receptors CXCR4 and CCR5 in HIV-1-infected and uninfected individuals. *Journal of Immunology.***161:**3195-3201.

Rodgriguez-Sosa M, Satoskar AR, Calderon R, *et al*. 2002. Chronic helminth infection induces alternatively activated macrophages expressing high levels of CCR5 with low IL-12 production and Th2-biasing ability. *Infection and Immunity.* **70 (7):** 3656-3664.

Secor WE, Shah A, Mwinzi PMN, *et al*. 2003. Increased density of human immunodeficiency virus type 1 coreceptors CCR5 and CXCR4 on the surfaces of $CD4^{+}$ T cells and monocytes of patients with *Schistosoma mansoni* infection*. Infection and Immunity*. **71 (11):** 6668-6671.

Shalekoff S, Pendle S, Johnson D, *et al*. 2001. Distribution of the Human Immunodeficiency Virus coreceptors CXCR4 aand CCR5 on leukocytes of persons with Human Immunodeficiency Virus type 1 infection and pulmonary tuberculosis: Implications for pathogenesis. *Journal of Clinical Immunology.* **21 (6):** 390-401.

Shalekoff S, Gray G, Tiemessen CT. 2004. Age related changes in expression of CXCR4 and CCR5 on peripheral blood leukocytes from uninfected infants born to Human Immunodeficiency Virus type 1-infected mothers. *Clinical and Diagnostic Laboratory Immunology.* **11 (1):**229-234.

Shang H, Zhang Z, Jiang Y, *et al.* 2005. Activation and coreceptor expression of T lymphocytes in HIV/AIDS patients of China. *Journal of Clinical Immunology.* **25(1):**68-72.

Sousa AE, Carneiro J, Meier-Schellersheim M, *et al*. 2002. CD4 Tcell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *The Journal of Immunology.* **169:**3400-3406.

Sozzani S, Ghezzi S, Iannolo G, *et al*. 1998. Interleukin-10 increases CCR5 expression and HIV infection in human monocytes. *Journal of Experimantal Medicine.* **187 (3):** 439-444.

Chapter 5

Proliferative and cytokine responses in HIV-1 positive and negative subgroups with or without helminth co-infection

5.1 Introduction

The simplified normal sequence of events during a stimulating antigen encounter by the immune system is: Stimulus → Immune activation →Differentiation into effector/memory cells→Stimulus eliminated→Immune response suppression/and activation-induced apoptosis. The last step of immune suppression mitigates against immune-induced pathological damage to tissues involved in the immune process (Janeway *et al.,* 2001; Maizels *et al*., 2004). It is mediated by regulatory/suppressor T cells (T regs) (Maizels *et al*., 2004; Diaz and Allen, 2007). These cells secrete or express on their surface downregulatory molecules, the prominent among them being CTLA-4, and the cytokines IL-10 and TGFβ (Borkow and Bentwich, 2004; Maizels *et al*., 2004; Diaz and Allen, 2007). Failure to eliminate the stimulating antigen results in abberration of the process, characterised by chronic activation, cellular dysfunction and suppression, plus increased activation-induced apoptosis (Lawn *et al.,* 2001).

In helminth infections the host fails to eradicate the antigen in the absence of chemotherapy. In HIV, even therapeutic intervention does not eliminate the antigen. This constant stimulation of the immune system is cited as the main cause of the quantitative immune dysregulation and cellular functional defects encountered in the two infections (Hazernberg *et al*., 2003; Borkow and Bentwich, 2004).

5.1.1 Cytokine and proliferative responses in helminth infection

The archetypal type 2 cytokine immune response to helminths is characterised by increased IL-4, IL-5, IL-9, IL-10, and IL-13. In addition to this universal response, helminth infections are accompanied by intricate processes of altered immune responses.

Firstly, helminth infection is associated with specific suppression of the immune responses directed against their antigens, for example, schistosomiasis, (Grogan *et al.,* 1996) and onchocercariasis (King *et al*., 1993) infected individuals had impaired

responses to schistosomal and filarial antigens respectively.This immune suppression then extends beyond antiparasite responses and becomes generalised to unrelated antigens (Actor *et al.*, 1993; Borkow and Bentwich, 2004; Maizels *et al.*, 2004).

Secondly the helminth-induced Th_2 polarised immune response spills over to bystander cells, influences the response to other non parasite antigens, and skews them to a Th_2 phenotype: In mice studies, *Nippostrongylus brasieliensis* infection was shown to induce differentiation of naïve, non-parasite specific T cells into Th_2 cells (Liu *et al.*, 2002). In humans, significantly impaired proliferative, IL-2 and intereferon γ responses have been documented in tetanus toxoid-vacinated, Onchocercariasis-infected children (Cooper *et al.*, 1998); *Ascaris lumbricoides*-infected adults vaccinated with cholera toxin (Cooper *et al.*, 2000; 2001) and BCG vaccinees infected with various helminths had attenuated T cell and skin test responses (Elias *et al.*, 2001). Neonates and infants sensitised in utero with schistosoma and filarial antigens showed 26-fold reduction of T cell intereferon γ responses to PPD compared to their unexposed counterparts (Malhotra *et al.,* 1999).

Lastly generalised anergy as a strategy for dampening the immune response directed against infecting parasites, is a feature of helminthiasis (Borkow and Bentwich, 2004). The increased expression of regulatory cytokines IL-10 and TGFβ, increased levels of the downmodulatory protein-CTLA-4 plus decreased levels of the costimulatory molecule CD28 contribute to the immune suppression and anergy during helminthiasis (Maizels and Yazdanbakhsh, 2003; Borkow and Bentwich, 2004). Both IL-10 and TGFβ have been shown to negatively correlate with T and B cell proliferation (Cooper *et al.*, 1998; Borkow and Bentwich, 2004). Proliferation of these cells in response to antigens would therefore be impaired.

As a result, this background Th_2 bias and immune suppression spill-over is suggested to influence the course of infection with other intracellular pathogens such as TB and HIV, whose control is largely dependent on Th_1 biased responses (Bentwich *et al.*, 1999). The attenuated responses to mycobacterial antigens and the general failure of BCG vaccination in sub-Saharan Africa and other developing countries have been attributed in part to helminth-induced immune dysregulation associated with helminth infection (Borkow and Bentwich, 2004).

5.1.2 HIV infection: cytokine and proliferative responses

During HIV infection, similar changes in immune function are reported, i.e generalised anergy, increased IL-10 and TGFβ and impaired proliferative responses (Borkow and Bentwich, 2004). HIV infection is also characterised by disruption of the cytokine networks. The initial response to the virus is increased levels of the antiviral Th_1 cytokine IFNγ and the lymphoproliferative IL-2 (Boaz *et al.*, 2002; Bahbouhi *et al.*, 2004). Literature suggests that as disease progresses these cytokines decline and an increase in proinflammatory cytokines TNFα, IL-1, IL-6 and IL-8, and the Th_2 cytokines IL-4 and IL-10 occurs (Kedzierska and Crowse, 2001). According to the popular and controversial Th_1/Th_2 theory, Th_1 (or type 1) cytokines are associated with protective cellular responses and good prognosis in HIV infection (Clerici and Shearer, 1994; Klein *et al.*, 1997). Furthermore, later studies confirmed the existence of $CD4^+$ T cells that simultaneously produce both type 1 cytokines (IFNγ and IL-2) that are associated with better control of HIV (Sousa *et al.*, 2001; Sieg *et al.*,2001; Boaz *et al.*, 2002).

Defective lymphoproliferative responses by HIV-specific lymphocytes are well documented (Palmer *et al.*, 2002; Borkow and Bentwich, 2004) and have been shown to correlate negatively with disease progression (Day *et al.*, 2007).

5.1.3 Hypothetical interactions between HIV and helminth infections

From the above, it is thus reasonable to expect that helminths may negatively influence the immune responses to HIV by further increasing impaired proliferation and skewing the response to a Th_2 phenotype. It is therefore hypothesised that infection by helminths compromises/influences immune responses to HIV as reflected by the type of cytokines produced and the quality of proliferative responses to viral antigens in dually infected individuals.

5.1.4 Objectives

The present chapter then evaluates cellular functional responses by means of (i) proliferation of PBMCs in response to stimulation with mitogen, recall antigens as well as HIV-1 p24 and *Ascaris lumbriciodes* antigens and (ii) determining the profile of cytokine production in unstimulated cells and in response to the specific antigens. The objectives were to determine if HIV and helminth coinfected individuals (i) secrete predominantly Th_2 cytokines, (ii) have impaired proliferative responses to mitogens and antigens compared to their helminth uninfected counterparts. The profile of inflammatory cytokines is also assessed. The first section evaluated proliferative responses as measured by the expression of the proliferation nuclear antigen Ki67 by flow cytometry. Simultaneous detection of the modulatory protein CTLA-4 was undertaken. The second section assessed the profile of cytokines by multiplex technology in HIV infected individuals with or without evidence of enteric parasite exposure. Owing to the costs of the multiplex cytokine analyses, the assay was undertaken for the HIV positive participants only.

5.2 Methods

Optimisation of culture conditions was first undertaken. The type of suitable mitogen (including Phytohaemagglutinin (PHA) or Phorbol myristate acetate/Calcium Ionomycin (PMA: IO) type of recall antigen (Purified Protein Derivative (PPD) or Streptokinase), the specific antigens (HIV-1 p24 and *Ascaris lumbricoides* crude antigen) and their optimum working concetrations were evaluated. Different incubation periods (6, 24, 48, 72 and 120 hours) were also assessed. IL-4 and IFNγ cytokine Elisa (R and D Inc.) were used for determination of optimal culture coditions. The full preparation of antigens, media and optimisation of the culture conditions are described fully in Appendix 6 page-254.
Finally PHA was used as a suitable mitogen and Streptokinase was selected over PPD because of its consistent results.The rationale for using streptokinase was based on its proven potency as a recall antigen (Youkeles *et al*., 1991) and in this work it was used as a positive control for proliferative capacity in response to recall antigen, while PHA was used for non-specific proliferation responsiveness.

5.2.1 *In vitro* culture and stimulation of PBMCs

5.2.1.1 Samples

PBMCs which had been frozen in liquid nitrogen were retrieved from cryopreservation as decribed in section 4.2.1. These cells were cultured in the presence of molecules associated with antigen presentation (anti-CD28 and anti-CD49d) without stimulation (designated unstimulated) and stimulated with mitogen and antigens following recommended standard laboratory methods. Forty samples of PBMCs from HIV-1 positive participants and 22 from HIV negative controls were set up in culture as described below.

5.2.1.2 Procedure

Cells were suspended at $1x10^6$ cells/ml in complete RPMI 1640 (cRPMI) with glutamax and supplements (anti-CD28 and anti-CD49d (BD), 1µg/ml each; 10% human male AB serum (Gibco); 2-mercaptoethanol (βME) 50µM; Penicillin 100U/ml and Streptomycin 100ug/ml (Gibco)). The cultures were set up with or without stimulants in 48-well polypropylene tissue culture plates in 1 ml total volumes. For all 40 HIV-1 positive participants, five wells per individual were assigned, one well containing medium and cells only (unstimulated) and four containing cell suspensions in cRPMI and the various stimulants. For the 22 HIV-1 negative participants, one unstimulated and three stimulated culture wells were assigned as shown in Table 5.1. The plate contents were mixed at 1000 rpm for 1 minute in a plate shaker and then incubated at 37ºC in a humidified 5% CO_2 incubator for 5 days. On day 5, without disturbing the cell pellets, 800 µl supernatants were collected into 4 sterile, appropriately labeled Eppendorf vials (each vial containing 200 µl of supernatant).The vials were centrifuged and placed into storage boxes and immediately transferred to a -70ºC freezer for later analysis of cytokine production (Section 5.2.3) and the cell pellets aliquoted for proliferation marker staining (Section 5.2.2).

Table 5.1 Culture plate setup for stimulation assays.
The different stimulants were added to 1x106 cells/ml of cRPMI in 48-well tissue culture plates for each sample assigned to the designated well. Samples for HIV-1 positive participants were distributed into 5 wells while those of HIV-1 negative participants were added into 4 wells.

	HIV-1 Positive	HIV-1 Negative
Stimulating Antigens	Unstimulated (cRPMI +cells)	Unstimulated(cRPMI +cells)
	PHA 5ug/ml (Sigma)	PHA 5ug/ml
	HIV-1 p24 5ug/ml (R&D Inc.)	
	Streptokinase 2ug/ml (Aventis)	Streptokinase 2 ug/ml
	A. lumbricoides 10 ug/ml	*A. lumbricoides* 10 ug/ml

A.lumbricoides= *Ascaris lumbricoides* crude antigen homogenate. Details of sources and preparation are in Appendix 6 page 254. PHA Phytohaemagluttinin; cRPMI complete RPMI medium

5.2.2 Determination of Proliferative Responses to Stimulants in cultured PBMCs: Ki67 intracellular staining

Preliminary experiments

A few methods were explored to identify a proliferation marker that would be suitable for the limited number of cells available for cultures. The two markers of proliferation, Ki67 antigen and Proliferating Cell Nuclear Antigen (PCNA) were found to be suitable. Poor and inconsistent staining was experienced with the latter and the former was then chosen for subsequent experiments. The procedure for Ki67 was further optimised to determine the best method for the flow cytometric recovery of stained (proliferating) cells.

5.2.2.1 Anti-Ki67 and anti- CTLA-4 staining

The final modified staining included staining for the downregulator of T cell stimulation, CTLA-4. Surface staining for CD3 expression was excluded as this staining proved unsuccessful due to poor light scatter properties of alcohol-preserved cells. PBMCs from 40 HIV-1 positive participants had each been cultured unstimulated or in the presence of PHA, HIV-1 p24, Streptokinase and *Ascaris lumbricoides* antigens. Control samples from 22 HIV-1 negative individuals were cultured unstimulated, or challenged with PHA, streptokinase and *A. lumbricoides* antigens. After removal of supernatants, the cell

cultures (from section 5.2.1.2) were enumerated and 500 µl aliquots adjusted to contain 5 $x10^6$ cells. For each sample, 5 wells of a 48-well microplate were designated for the respective stimulant (in duplicates) as listed in Table 5.1. Twenty microliters each of monoclonal antibody-fluorochrome conjugate (CTLA-4 APC) were carefully pipetted into the bottom of all designated wells. One hundred microliter aliquots of each sample were pipetted into each of the corresponding wells containing the antibodies. The solutions were mixed by shaking the plates in the shaker at 1000rpm for 30 seconds. Plates were then incubated on ice for 45 minutes in the dark. For washing, the plates were then centrifuged at 1500rpm with the brake on at 4ºC for 3 minutes and supernatant carefully aspirated. The wash/centrifugation step was repeated one more time. The pelleted cells/ 100 µl were transferred to 50ml polypropylene tubes. Five milliliters of cold 80% ethanol was added dropwise, while vortexing the cell suspensions. The cells were then fixed and permeabilised by incubation for at least 2 hours at -20ºC (in most cases were incubated overnight- they could be used up to 60 days after fixing). Forty milliliters of wash buffer (1X PBS with 1% fetal calf serum and 0,09% NaN_3, pH 7.2) was added to the fixed cells and the tubes centrifuged at 1000 rpm (with the brake on) for 10 minutes. The supernatant was carefully aspirated and discarded. A further wash-centrifugation step was repeated two more times and all supernatant completely removed. The cells were resuspended in 100 µl of wash buffer and transferred to designated wells containing 20 µl of anti Ki67-FITC conjugate (BD Pharmingen) in 48-well microtiter plate. The sample-antibody solutions were mixed in a plate shaker at 1000 rpm for 30 seconds and incubated on ice for 45 minutes in the dark. One hundred and eighty microliters of wash buffer was dispensed into each well and the plates centrifuged as above. The washing steps were repeated once more. The pellets were resuspended in 180 µl of wash buffer and transferred to FACS tubes containing 350µl of wash buffer. Flow cytometric analysis of the samples then followed. Control samples wells were included: IgG1 κ FITC and IgG_2a APC isotype controls, unstained cells, stimulated and unstimulated cells stained with anti-Ki67 FITC only, anti-CTLA-4 APC only, and with both sets of antibodies.

5.2.2.2 Flow cytometric detection: Data Acquisition and analysis

The daily instrument standardization and maintenance was performed. Using the Cellquest software for four colour analysis, the unstained cells were first run through the

instrument for the negative autoflourescence control and threshold setting. Forward and side scatter detector PMT voltage and Amp gain settings were also adjusted using the unstained cells tube. The threshold value was set at 180 on forward scatter to exclude debris. Cells stained with isotype controls were used to exclude non-specific binding of antibodies. The following isotype controls were included: IgG_1k FITC and IgG_2a APC. Further, unstained cells from a HIV negative and helminth uninfected individual were finally used to set the negative population marker as isotype control tubes were found unsuitable for this. The samples were then run on Cellquest software. A minimum of 10 000 events were acquired for each sample tube in list mode.

Gating.

In the side scatter versus forward scatter dot plot, the lymphocyte region (R1) was established. For each participant, the unstimulated tube was used to set the gating region and the curser was left unchanged for all subsequent tubes of stimulated cells for the particular individual. Thus the R1 lymphogate region of the unstimulated cells tube was also used for gating the stimulated cells for each individual. Dotplots for Ki67 vs CTLA-4 were drawn and histogram plots were drawn with markers separating the negative and positive cell populations, each for Ki67 and CTLA-4 fluorescence intensity as in Figure 5.1

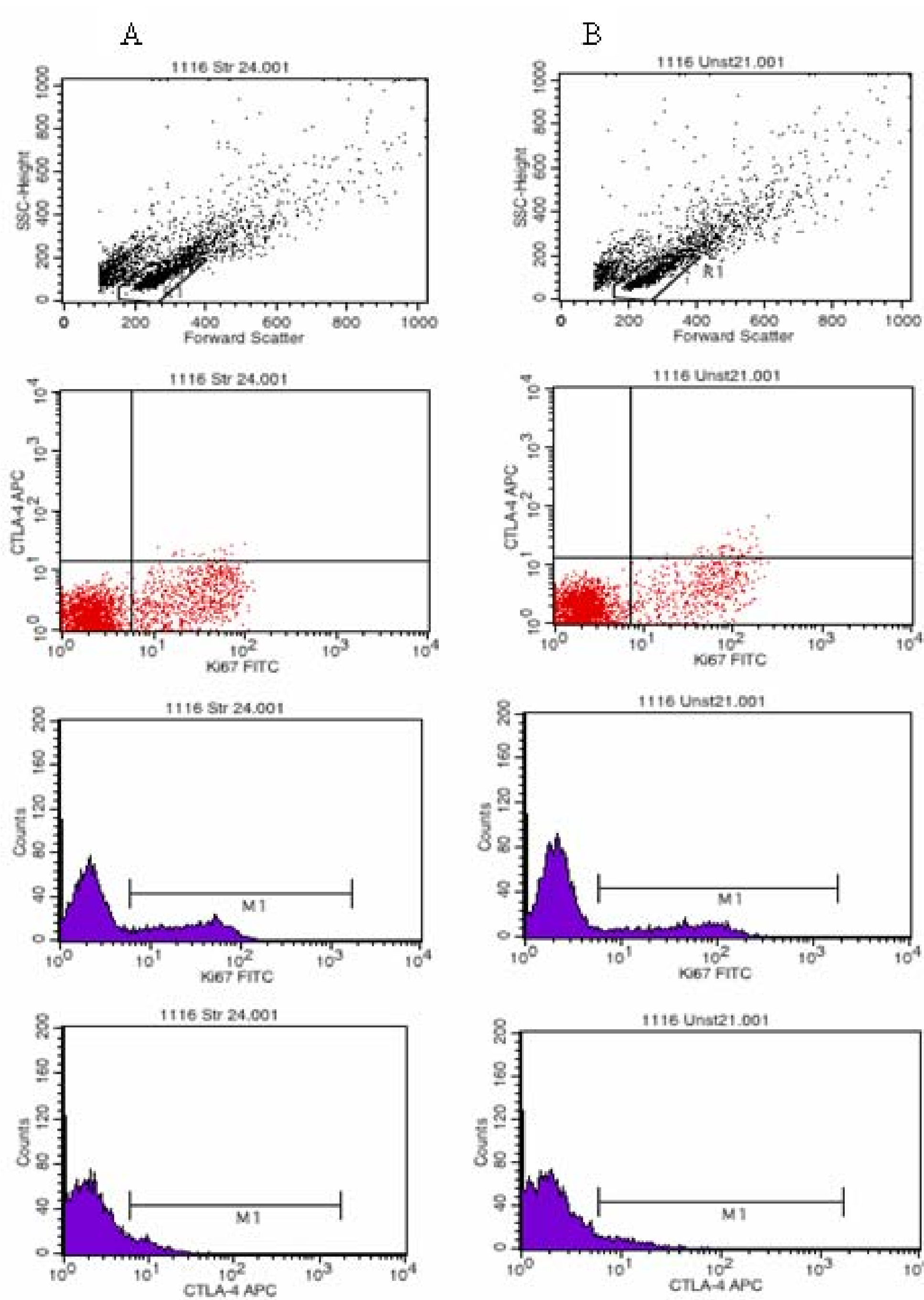


Figure 5.1 Gating strategy for Ki67 and CTLA-4 expression by lymphocytes.
PBMCs were cultured in RPMI medium without stimulation and with HIV-1 p24, PHA, *Ascaris lumbricoides*, or Streptokinase challenge for 5 days. These cells were fixed, permeabilised, stained with anti-Ki67 FITC and anti-CTLA-4 APC conjugates. Expression of these proteins was analysed on gated lymphocytes by flow cytometric determination of fluorescence intensity. A. Side scatter vs forward scatter properties were used to differentiate lymphocytes in region 1(R1) B. The unstimulated cells tube B (upper right) was used to set the curser, and then the stimulated cells (streptokinase in this example) were gated on the same region. All subsequent stimulated cells were gated on the same region set on unstimulated cells tube for each individual.

5.2.3 Detection of cytokine production by PBMCs

5.2.3.1 Methodology-Multiplex Human Cytokine Assay

For these tests 40 samples from HIV infected participants were analysed. Cell culture supernatants that had been harvested after five days' culture (section 5.2.1.2) were retrieved from -70°C freezers and allowed to cool to room temperature before assays were set up. The Human cytokine LINCOplex KIT (13-plex) assay was used as per the manufacture's instructions to detect a range of thirteen cytokines. Briefly, plates were BSA- blocked and washed. Cytokine standards, controls and unknown samples were added (50µL total volumes) to 96 well plates in duplicates and incubated with antibody-immobilised micro beads. After washing, biotynilated detection antibodies were incubated with the bound cytokines. Fluorescent (Phycoerythrin- labeled) streptavidin was added. A final wash was followed by resuspension in sheath fluid for analysis in the Bioplex array reader (Biorad) using the Bioplex Manager 4.1 software. Fifty beads per region were collected. A seven-point standard curve (range 0; 3,2; 16; 80; 400; 2000 and 10 000pg/ml) was constructed using a 5 parameter logistic (5PL) regression and the concentration of each cytokine calculated against this curve.

5.2.4 Statistical methods

The median was used and Kruskal Wallis ANOVA was used to compare the differences between the subgroups.

5.3 Results

The proliferative capacity and cytokine production of cells from HIV infected and uninfected persons with or without evidence of helminth exposure were measured. PBMCs were incubated for five days with or without stimulation and proportions of cells expressing the proliferation nuclear antigen Ki67 and downregulatory molecule CTLA-4 determined by flow cytometry. Culture supernatants were analysed for cytokine production by multiplex (Bioplex, BioRad Inc) technology.

5.3.1 Results of Preliminary experiments

Optimum concentrations of stimulants were 5ug/ml each for PHA and HIV-1 p24, 2ug/ml for streptokinase and 10ug/ml for the Ascaris crude antigen preparation. The suitable

incubation period was found to be five days particularly for the antigens, although PHA responses had already peaked on day 3 for IL-4 production while interferon γ peaked at day five for specific antigens. It became clear that optimum culture conditions differed for PHA and specific antigen as well as for the two cytokines IL-4 and IFNγ, and a compromise was a five day culture, because the more important outcome was for antigen-specific responses.

5.3.2 Proliferation Assay Results: Unstimulated (baseline) and stimulated proliferation responses to mitogen and recall antigens: Ki67 and CTLA-4 expression in HIV^+ and HIV^- groups:

Background (unstimulated) values were subtracted from the values obtained in each antigen-specific response. There were indeed differences in the expression of Ki67 in the absence of stimulation and also in response to stimulants between the two HIV groups, with a significantly lower Ki67 median values in HIV negatives than HIV positives in unstimulated cells (p=0.01). Also, within the subgroups, there were differences in the levels of background Ki67 and these are compared separately from the net antigen-specific responses. The median percentage expressionof Ki67 and CTLA-4 within the subgroups are summarized in Figure 5.2 for HIV positive and negative groups. Antigen specific responses were generally higher in the HIV negative group compared to the HIV positive groups (Figure 5.2 panels B and D) Streptokinase p=0.05 and Ascaris p=0.29). These differences in antigen-specific proliferative capacity can possibly be ascribed to the effect of HIV infection. It should be noted that the numbers of some of the HIV negative subgroups were smaller than those of the HIV+ groups. The finding of higher antigen responses despite smaller numbers in this group is noted. However for statistical inferences all comparisons involving the HIV negative subgroups need to be considered with caution.

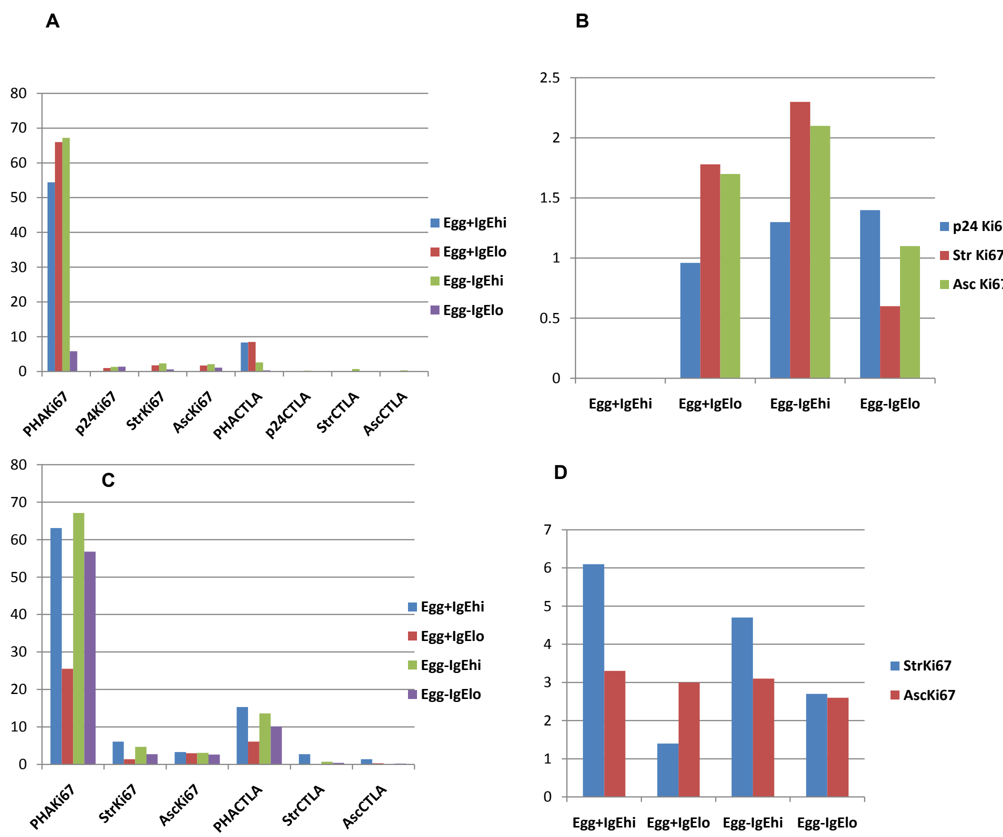


Figure 5.2 Median percentage expression of proliferation nuclear antigen (Ki67) and Cytotoxic T lymphocyte antigen-4 (CTLA-4) in lymphocytes in HIV+ (A and B) and HIV- subgroups (C and D) with or without helminth co-infection. A and C represent summary Ki67and CTL-A4 net responses to all stimulants while B and D show net Ki67 responses to specific antigens in HIV+ and HIV- groups respectively. PBMCs were incubated unstimulated or stimulated with PHA, *Ascaris lumbricoides* and Streptokinase antigens. After five days of culture, cells were stained with monoclonal antibodies to Ki67 and CTLA-4 and proportions of stimulated or unstimulated cells expressing these markers were determined by flow cytometry. Median values in the y axis are expressed as percentages. In all panels the groups and their n sizes were: $Egg^{+}IgE^{hi}$ group (HIV+ n = 10,HIV- n=5**)** helminth egg excretion and elevated *Ascaris lumbricoides* IgE; $Egg^{+}IgE^{lo}$ (HIV+n=10, HIV-n=7) helminth egg excretion and low Ascaris *lumbricoides* IgE; $Egg^{-}IgE^{hi}$ group (HIV+n=10, HIV-n=3); $Egg^{-}Ig^{lo}$ (HIV+n=10, HIV-n=7) No helminth egg excretion and low *Ascaris lumbricoides* IgE. PHA= Phytohaemagluttinin-;Str Streptokinase-p24 HIV p- 24 -stimulated cells.

5.3.3 Proliferation profile within subgroups of HIV positive and negative subgroups

The comparison of proliferative capacity between the subgroups of HIV positive individuals was done by Kruskal Wallis Anova and subgroup medians are summarised in Figure 5.2

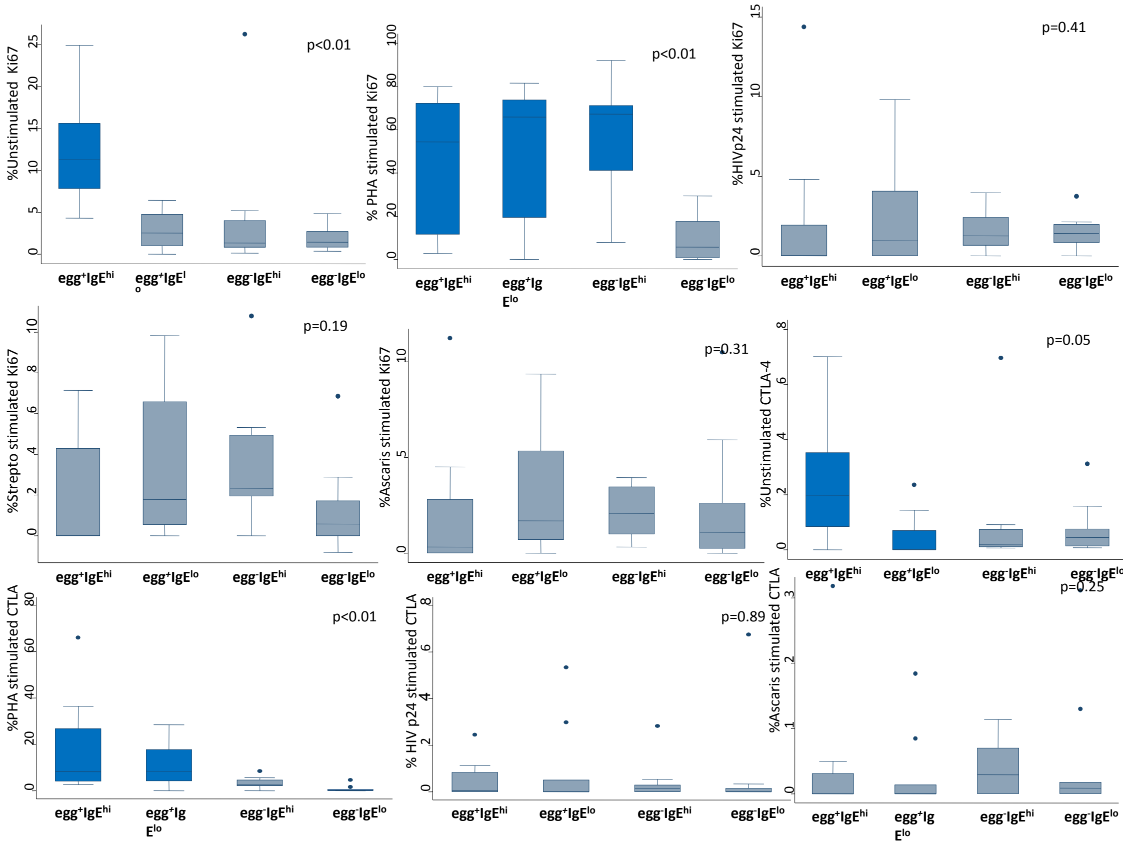


Figure 5. 3 Kruskal Wallis Box and whisker plots for proliferation antigen - Ki67 and CTLA-4 antigen expression on unstimulated lymphocytes of HIV+ subgroups.
Lymphocyte expression of Ki67 and CTLA-4 was determined by flow cytometry. The median percentages were compared by Kruskal Wallis ANOVA to determine significant differences within the subgroups of HIV^+ individuals. Ascaris-crude whole worm antigen of *Ascaris lumbricoides.* Its preparation is detailed in Appendix 6.1 page 254. Significantly different groups are shown in deep blue.

5.3.4. Interactions of proliferation profiles in subgroups of HIV positive and negative subgroups.

5.3.4.1 The HIV+, egg^{+}IgE^{hi} group is associated with increased expression of Ki67 proliferation antigen in unstimulated cells.

In HIV infected, egg^{+}IgE^{hi} participants, expression of Ki67 in unstimulated was significantly higher (p=0.01) cells than in the egg^{-}IgE^{lo} and the other two subgroups. In relation to the other HIV^{+} subgroups, the median Ki67 expression by unstimulated cells was up to four to eight times higher in this subgroup compared to the egg^{-}IgE^{lo} (p <0.01), the egg^{+}IgE^{lo} (p=0.01), and the egg^{-}IgE^{hi} (p=0.01) subgroups Furthermore, in most instances, the expression of Ki67 was higher in unstimulated than in antigen-stimulated cells in the egg^{+}IgE^{hi} subgroup (Figure 5.4). Subsequently, the median percentage values for the antigen responses equaled zero for all specific antigens (Figure 5.2 panel B).

The background values were then subtracted from the values obtained in the antigen stimulated cells.

The HIV-, egg^{+}IgE^{hi} and egg^{-}IgE^{lo} subgroups

There were no significant differences in expression of Ki67 in unstimulated cells when the HIV negative, helminth infected individuals were compared to the helminth uninfected subgroup. However, the median percentage Ki67 was slightly but insignificantly higher in the egg^{+}IgE^{hi} subgroup (median 4,3%, compared to 1,1%, 1,0% and 2,6% respectively for the other three subgroups).

In all the HIV negative subgroups almost all background (unstimulated) values were lower than stimulated ones.

5.3.4.2 The egg^{+}IgE^{hi} group shows a tendency towards impaired proliferative responses to recall and specific antigens but potent mitogen responses.

In the HIV/helminth co-infected, egg^{+}IgE^{hi} subgroup, there were consistenly low antigen–specific (HIV-1 p24 and Ascaris) and recall antigen (Streptokinase) Ki67 responses

relative to the egg^{-}IgE^{lo} and all the other subgroups (Figure 5.2 panel B) while mitogen responses remained high (Figure 5.2 panel A). These differences were however not significant (p=0.41, 0.19 and 0.31 for p24, streptokinase and Ascaris, respectively). Of note is the fact that compared to the HIV-singly infected individuals, the egg^{+}IgE^{hi} subgroup had undetectable levels with zero median Ki67 responses to all specific antigens while these were 1.4%, 0.6% and 1.1% for p24, streptokinase and Ascaris antigens respectively in the HIV+ egg^{-}gE^{lo} subgroup.

Notably, the strong Ki67 responses to PHA mitogen were highly significant (p=<0.01) in all groups with some evidence of helminth exposure (the egg^{+}IgE^{hi}, the egg^{+}IgE^{lo} and the egg^{-}IgE^{hi} groups) compared to the egg^{-}IgE^{lo} group (Figure 5.2 panel A)

In this (egg^{+}IgE^{hi}) subgroup, most participants had enhanced PHA mitogen proliferative responses while HIV-p24 and Ascaris antigen responses were decreased (Figure 5.5) in this egg^{+}IgE^{hi} group). It is noted however that some individuals within this group made strong antigen-specific responses (Figure 5.6)

The HIV-, egg^{+}IgE^{hi} group

Among the HIV uninfected groups, antigen specific responses were similar for the egg^{+}IgE^{hi} and all the other three subgroups, particularly for the Ascaris stimulated Ki67 expression responses.

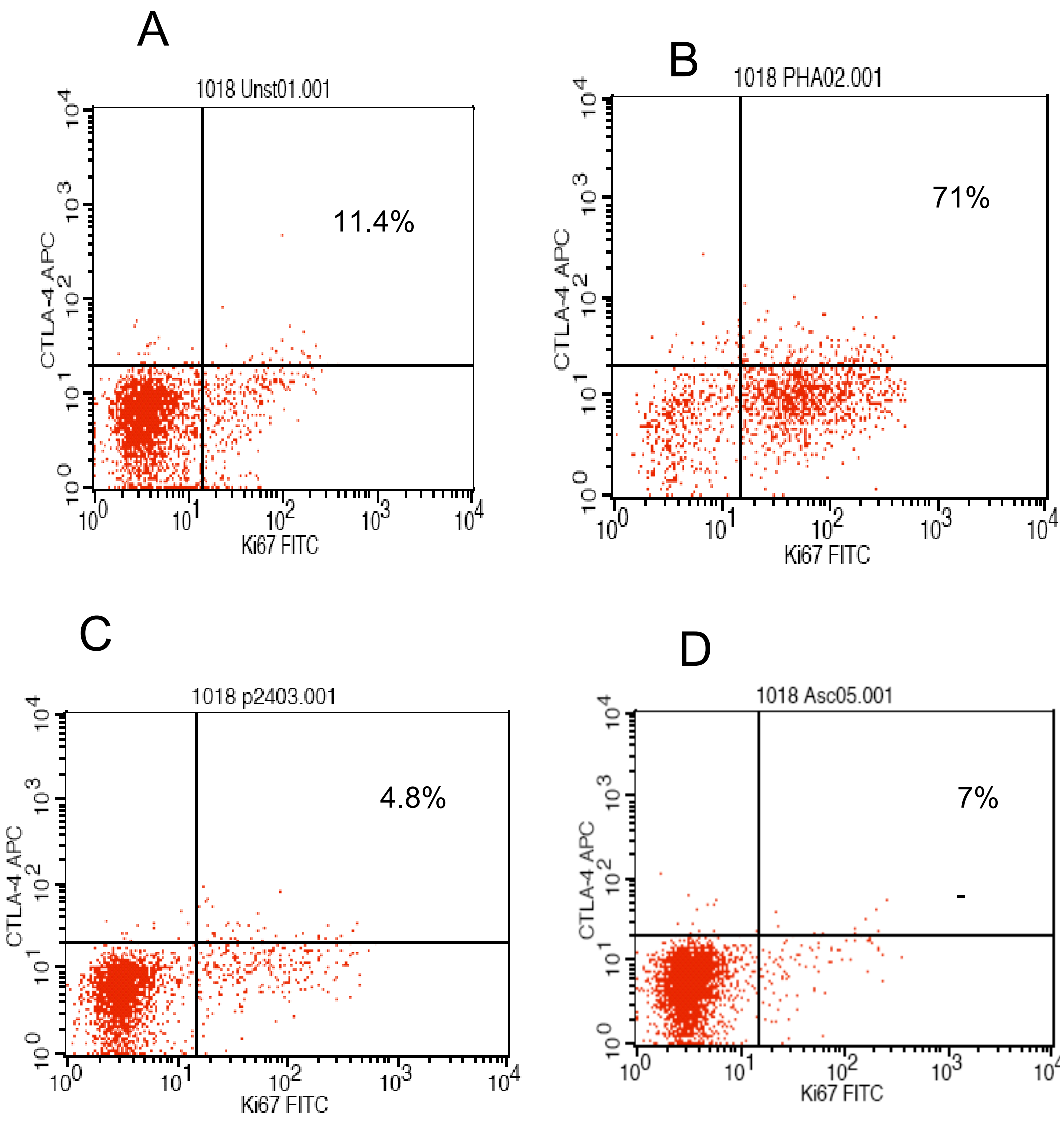


Figure 5.4 Illustrates increased expression of Ki67 on unstimulated cells. Cells cultured without stimulation (Panel A) show increased expression of KI67 compared to HIV-p24 (panel C) and Ascaris-antigen (panel D)-challenged cells in an HIV-1 infected, egg excreting individual with elevated *Ascaris* IgE. PBMCs were stained with monoclonal antibodies to Ki67 and CTLA-4 conjugated to FITC and APC respectivelyand analysed for proportions of cells expressing these molecules by flow cytometry. Percentages in this figure represent all cells expressing Ki67 (top and bottom right quadrants).

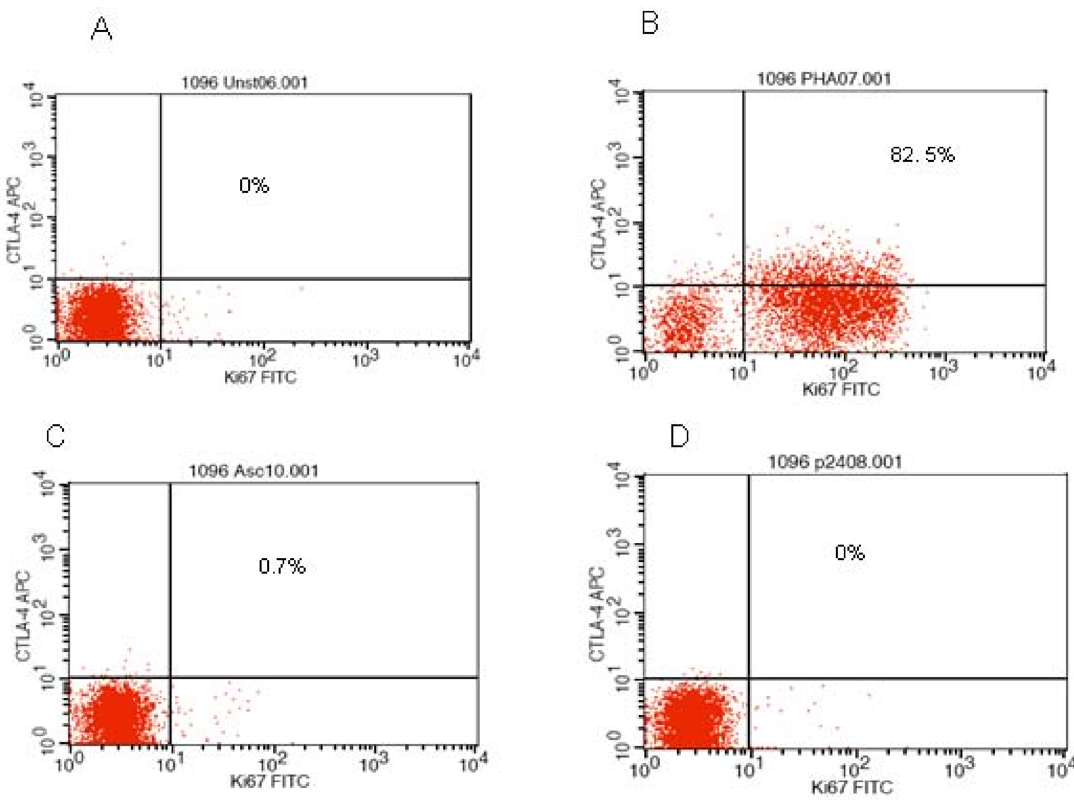


Figure 5.4 Increased mitogen and low specific-antigen responses in an HIV+ Egg^{+}IgE^{hi} individual. Enhanced PHA responses (panel B) compared to *Ascaris* (panel C) and HIV-1 p24 (panel D) specific antigen responses in an HIV+ individual with egg^{+}IgE^{hi} status. PBMCs were stained with monoclonal antibodies to Ki67 and CTLA-4 conjugated to FITC and APC respectively and analysed for proportions of cells expressing these molecules by flow cytometry. Percentages in this figure represent all cells expressing Ki67 (top and bottom right quadrants).

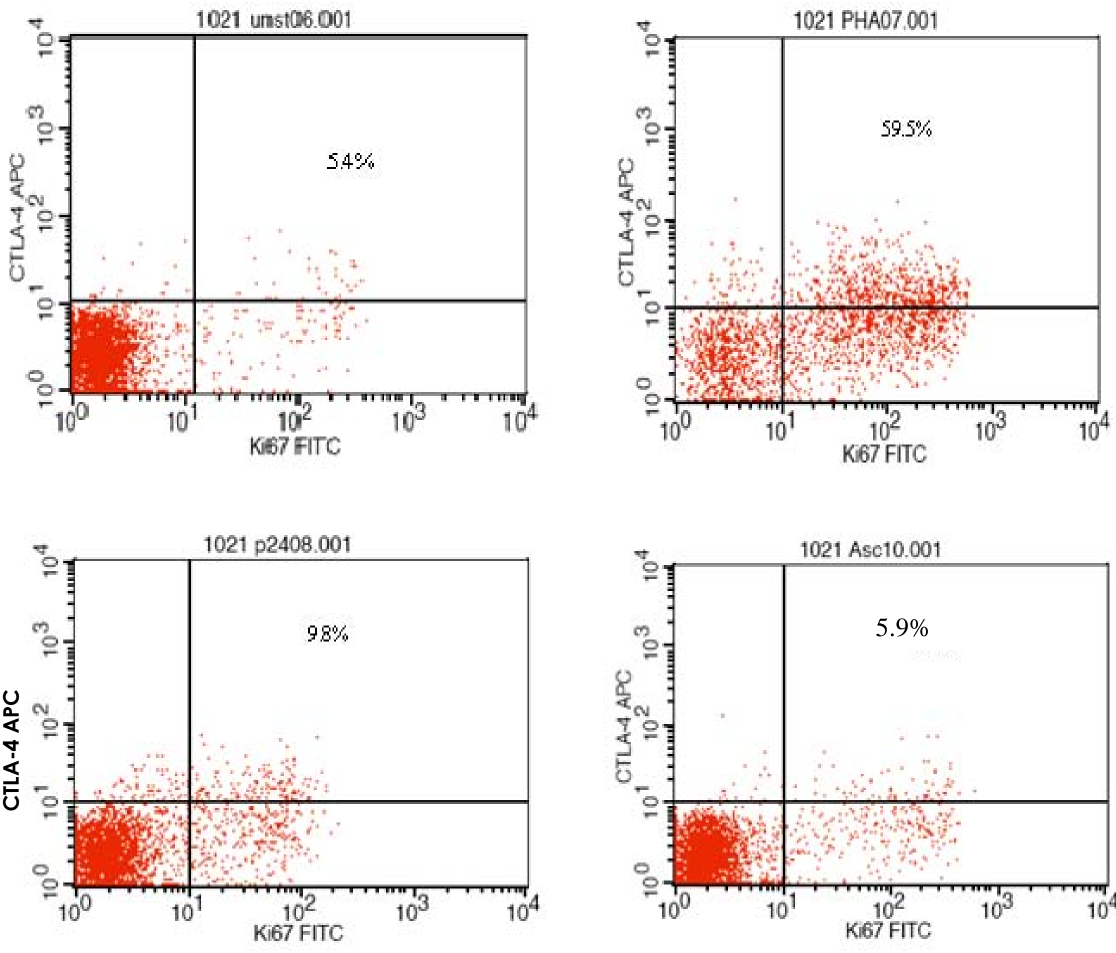


Figure 5.5 Potent antigen specific responses (HIV-p24, panel C and *Ascaris* antigen, Panel D) from an HIV positive–Egg^{+}IgE^{hi} helminth infected individual.
PBMCs were stained with monoclonal antibodies to Ki67 and CTLA-4 conjugated to FITC and APC respectively and analysed for proportions of cells expressing these molecules by flow cytometry. Percentages in this figure represent all cells expressing Ki67 (top and bottom right quadrants).

5.3.4.3 CTLA-4 Responses

5.3.4.3 The HIV+ $egg^{+}IgE^{hi}$ group is associated with increased CTLA-4 expression in unstimulated cells

The downregulatory protein CTLA-4 in unstimulated cells in the $egg^{+}IgE^{hi}$ group did not differ with that of the $egg^{-l}gE^{lo}$ group but was significantly higher in relation to the $egg^{+}IgE^{lo}$ subgroup (p=0.04;

Notably CTLA-4 expression in response to PHA challenge was also statistically significantly higher ($p<0.001$) in both groups with stool eggs (the $egg^{+}IgE^{hi}$ and $egg^{+}IgE^{lo}$ groups- Figure 5.2 panel A). Low or undetectable levels of this molecule were observed for antigen specific stimulated cells in all the HIV+ subgroups.

The HIV- $egg^{+}IgE^{hi}$

CTLA-4 responses were significantly increased for Ascaris and streptokinase stimulated cells in the $egg^{+}IgE^{hi}$ subgroup (p=0.03 and 0.05 respectively) Figure 5.2 panel C. No significant increases in CTLA-4 were observed in the other subgroups.

Two other observations made in the HIV- groups:

1) Contrary to the responses observed in the HIV infected participants, the background Ki67 expression was significantly higher (p=0.03) in the helminth-negative $egg^{-}IgE^{lo}$ subgroup (, Figure 5.5 in Appendix 5.3) which probably suggests that these individuals have some background immune response to some antigenic challenge.

2) Among the HIV negative participants, both groups with high IgE had significantly higher proliferation responses to the recall antigen Streptokinase (Figure 5.2 Panel C). This could reflect that high IgE possibly decreases the threshold for immune responsiveness to recall antigens.

It should be noted that in both HIV positive and negative groups, some responses to Ascaris antigen challenge were made by the groups without faecal eggs and low specific IgE ($egg^{-}IgE^{lo}$) (medians 1.1% and 2.64% in HIV^{-} and HIV^{+}, respectively) These may suggest that there is some level of exposure to Ascaris antigens in the environment, or reflects past exposure/cleared past infection.

5. 3.7 Results of Cytokine Production Patterns

According to the theoretical interactions between HIV and helminths, type 2 cytokines induced by helminths would decrease the Th_1 cytokines that are essential for HIV control. On the other hand, HIV disease progression is cited to be associated with increased Th_2 cytokines or reduction in Th_1 response (Clerici and Shearer, 1994). In order to determine if HIV and helminth co-infected individuals present a predominance of type 2 cytokine profile and/or diminished type 1 cytokines, baseline cytokine production was compared in HIV^+ subgroups with and without evidence of helminth exposure. Proinflammatory cytokine profiles were also assessed as they are cited to increase as HIV progresses (Valdez and Lederman, 1997). Furthermore, cytokine responses to stimulation with HIV-1 p24 and *Ascaris* worm antigens were analysed.

5. 3.7.1 Cytokine production by unstimulated cells

The median values of baseline (unstimulated) cytokine production are shown in Table 5.2. A wide range of individual levels was detected,therefore prominent patterns are reported in the respective groups. Low background IL-5 and IL-13 levels were generally obtained in all the groups while IL-7 levels were undetectable. The level of expression of IL-1RA in particular, and to an extent IL-6 and IL-8 was very high, and many values for these cytokines were extrapolated outside the standard curve upper limit of 10 000 pg/ml (Table 5.2).

Table 5.2 Unstimulated Cytokine Production in HIV+ subgroups
.Median percentage expression of extracellular cytokines in unstimulated cell cultures in HIV infected groups with or without helminth co-infection. PBMCs at a concentration of 1x10^6 /ml of cRPMI were cultured without stimulation at 37°C for five days and supernatants were collected, centrifuged and stored at -70°. Later the supernatants were analyzed by the multiplex technology Bioplex (BioRad) for cytokine expression.

Cytokine(pg/ml)		**Egg$^+$IgE^{hi} N=10**	**Egg$^+$IgE^{lo} N=10**	**Egg$^-$IgE^{hi} N=10**	**Eggs$^-$IgE^{lo} N=9**
			Proinflammatory		
IL-6	**Median**	**10762**	**9157.6**	**10252.9**	**12670.5**
	Min	9.2	4065.9	191.9	>10000
	Max	12043.6	12984.8	12547.3	>10000
IL-8	**Median**	**8666.1**	**8652.8**	**10722.9**	**11934.0**
	Min	604.7	5926.8	6785.2	7936.1
	Max	10845.0	11872.4	11993.6	13129.8
TNFα	**Median**	**152.7**	**200.3**	**68.3**	**80.9**
	Min	0.46	18.7	22.6	16.2
	Max	468.5	1011.6	2759.1	397.8
			Anti-inflammatory		
IL-1RA	**Median**	**5369.0**	**6058.0**	**>10000**	**>10000**
	Min	49.7	2358.9	426	282.8
	Max	>10001.0	>10001.0	>10000	>10000
			Th$_1$		
IL-2	**Median**	**0**	**1.4**	**0**	**1.09**
	Min	0	0	0	0
	Max	0	6.84	5.82	5.94
IFNγ	**Median**	**0**	**2.0**	**1.2**	**0.4**
	Min	0	0	0.12	0
	Max	1.14	252.3	808.2	3.48
IP-10	**Median**	**40.9**	**174.0**	**47.8**	**112.8**
	Min	4.0	12.4	4.7	0
	Max	118.2	7738.8	1050.5	639.6
GM-CSF	**Median**	**104.9**	**85.1**	**46.9**	**53.5**
	Min	0	15.2	17.8	8.2
	Max	177	475.8	1997.2	291.9
			Th$_2$		
IL-4	**Median**	**19.5**	**9.6**	**20.2**	**15.6**
	Min	2.8	2.5	10.0	12.8
	Max	21.0	22.9	48.1	17.7
IL-5	**Median**	**0.05**	**0.08**	**0.16**	**0.1**
	Min	0.03	0.05	0.08	0.08
	Max	0.12	26.7	232.3	0.16
IL-13	**Median**	**0**	**0.16**	**3.71**	**0**
	Min	0	0	0	0.
	Max	10.77	161.4	2943	0
			Regulatory		
IL-10	**Median**	**30.7**	**116.3**	**40.0**	**146.6**
	Min	0.0	28.9	3.8	3.0
	Max	279.9	405.8	403.0	801

*Eggs- faecal helminth eggs. Hi /lo IgE- high or low serum *Ascaris lumbricoides*-specific IgE. IL interleukin; IL-1RA interleukin-1 receptor antagonist; IFNγ interferon gamma;IP-10 interferon-inducible protein 10; TNFα Tumor necrosis factor alpha; GM-CSF granulocyte-monocyte stimulation factor; RPMI=complete RPMI medium. Values extrapolated outside the standard curve's range are given as greater than 10 000.

5.3.8 Patterns of cytokine production by unstimulated cells in HIV^+ subgroups

5.3.8.1 Cytokine profiles in the egg^+IgE^{hi} and the egg^-IgE^{lo} subgroups

The egg^+IgE^{hi} subgroup had significantly lower levels of the anti-inflammatory IL-1RA ($p=0.04$), and a lower trend for the Th_1 cytokines IL-2 ($p=0.07$) and IFNγ ($p=0.09$) and all the Th_1 cytokines except for the GM-CSF were lower but not significant when compared to the egg^-IgE^{lo} subgroup, while both groups had similar proportions of proinflammatory cytokines but IL-8 was significantly lower ($p<0.01$) and TNFα higher, but not significant in the egg^+IgE^{hi} individuals.The Th_2 cytokines were similar in both groups, The regulatory IL-10 was lower but not significant in the egg^+IgE^{hi} group Table 5.2.

5.3.8.2 Type 1 cytokines are decreased in the egg^+IgE^{hi} group

All individuals in the egg^+IgE^{hi} group had undetectable baseline levels of the lymphoproliferative cytokine, IL-2 and a trend between this group and the egg^-IgE^{lo} group ($p=0.07$) was observed.

Similarly, in the egg^+IgE^{hi} group the antiviral cytokine, interferon gamma (IFNγ) was undetected in all except only one participant with 1,14pg/ml IFNγ. Median IFNγ production by unstimulated cells differed significantly between this group and the egg^+IgE^{lo} ($p=0.02$) and the egg^-IgE^{hi} ($p=0.04$) groups and there was a trend for a decrease in this group when compared to the egg^-IgE^{lo} group ($p=0.09$)

5.3.8.3 The Th_2 cytokine IL-4 is higher while Th_1 cytokines (IL-2 and IP-10) and the regulatory cytokine IL-10 are lower in high IgE responder (egg^+IgE^{hi} and egg^-IgE^{hi}) sugroups.

The median expression of the classic Th_2 cytokine, IL- 4, was highest in the two groups with elevated Ascaris IgE (egg^+IgE^{hi} and the egg^-IgE^{hi} groups) Table 5.2. This difference was statistically highly significant between the egg^-IgE^{hi} and the egg^+IgE^{lo} groups ($p=0.01$) and between the egg^-IgE^{hi} and the egg^-IgE^{lo} groups ($p=0.02$).In addition, while all participants in the egg^+IgE^{hi} group had undetectable IL-2 levels, this cytokine was produced by unstimulated cells in only 1 of the 10 egg^-IgE^{hi} participants, such that median IL-2 levels were marginal in both groups with high IgE (Table 5.2), and there was a weak trend between the egg^+IgE^{hi} and the egg^-IgE^{lo} groups ($p=0.07$)Table 5.2).

Table 5.2 shows that the levels of the interferon inducible protein (IP)-10 were also lower in these high IgE groups (lowest in the $egg^{+}IgE^{hi}$ group) compared to the low IgE responders (the $egg^{+}IgE^{lo}$ and $egg^{-}IgE^{lo}$ groups).

Furthermore, median values for the regulatory cytokine IL-10 were lowest in the 2 groups with high *Ascaris* IgE (medians 30.7 and 40 pg/ml) compared to the low IgE groups (medians 116.3 and 146.6 pg/ml).

5.3.8.4 The $egg^{+}IgE^{lo}$ individuals have higher IFNγ (type-1), and lower IL-4 (type 2) cytokines.

The $egg^{+}IgE^{lo}$ group had the highest median IFNγ, and this was statistically highly significant compared to the $egg^{+}IgE^{hi}$ (p=0.01) and the $egg^{-}IgE^{hi}$ groups (p<0.01) Table 5.2. Notably, the medians for IL-2 and IP-10 (Th_1) were also highest in this group (Table 5.2).

The median value for IL-4 was lowest in this $egg^{+}IgE^{lo}$ group and this was highly significantly lower compared to the $egg^{-}IgE^{hi}$ and the $egg^{+}IgE^{hi}$ groups (p=0.01.

The regulatory cytokine IL-10 was higher (but not significantly, p=0.16) in this subset of individuals compared to both high IgE responder groups.

5.3.8.5 Proinflammatory and anti-inflammatory cytokine production by unstimulated cells.

The proinflammatory cytokines IL-6 and IL-8 and the anti-inflammatory interleukin-1 receptor antagonist (IL-1RA) were highly expressed in most of the samples. This was more often the case in IL-1RA where all the values in the $egg^{-}IgE^{lo}$ and in five of the ten individuals in the $egg^{-}IgE^{hi}$ group far exceeded the upper curve limit and could not even be extrapolated. For both IL-6 and IL-8 most values were extrapolated outside the standard curve upper limit therefore data for these three cytokines may not be accurate. However as the results stand, there were two significant observations in the secretion patterns of these cytokines.

Firstly, the two groups with positive stool eggs ($egg^{+}IgE^{hi}$ and the $egg^{+}IgE^{lo)}$ had remarkably lower levels of IL-8. These differences were statistically highly significant

between the $egg^{+}IgE^{hi}$ and the $egg^{-}IgE^{lo}$ (p=0.01) and between the $egg^{+}IgE^{lo}$ and the $egg^{-}IgE^{lo}$ ($p<0.01$) groups

Secondly the anti-inflammatory cytokine IL-1RA was also lower in both stool egg positive groups, with a significant difference between the egg^{+} IgE^{hi} and the $egg^{-}IgE^{lo}$ groups (p=0.04 Table 5.6).

Finally, as the secretion levels of the other proinflammatory cytokine tumor necosis factor (TNF) α were within the curve plot range, it was observed that the two groups with positive helminth stool eggs ($egg^{+}IgE^{hi}$ and the $egg^{+}IgE^{lo}$) had higher medians for TNFα compared to the stool egg negative groups ($egg^{-}IgE^{hi}$ and the $egg^{-}IgE^{lo}$) (Table 5.2) although this difference did not reach statistical significance.

In summary the stool egg positive groups had higher levels of TNFα and lower levels of IL-1 RA and IL-8.

5. 3. 9 Cytokine production in antigen-stimulated cells

For the final antigen–specific results, the background (unstimulated) cytokine production values were subtracted from the specific result for each sample.

5.3.9.1 Cytokine production in HIV-1 p24-challenged cells

It was observed that cytokine responses to HIV-1 specific antigen challenge were distinctly lower than background (unstimulated) levels. This was particularly the case with the proinflammatory cytokines (IL-6, IL-8 and all the type 2 cytokines (IL-4, IL-5 and IL-13 (Table 5.3).

5.3.9.2 Comaprison between the dual infected HIV+ $egg^{+}IgE^{hi}$ and HIV singly-infected $egg^{-}IgE^{lo}$subgroups

Comparison between the $egg^{+}IgE^{hi}$ and $egg^{-}IgE^{lo}$ subgroups shows that the Th_1cytokine IP-10 was significantly lower (p=0.04) in the $egg^{+}IgE^{hi}$ individuals while IL-2 and GM-CSF were undetected in both groups. All the pro-inflammatory cytokines were higher, but not significant in the former group in HIV p24 stimulated cultures (Table 5.3). The IL-10 levels were similar in both groups. Th_2 cytokines were not detectable after stimulation of cultures with HIV p24 in the current assay except in a very few individuals in all subgroups. No other significant differences were observed between the dually infected and HIV-singly infected subgroups for the HIV antigen stimulations.

5.3.9.2 The Th1 cytokines (IL-2 and IFNγ) are lower in HIV-1 p24 stimulated cells of the $egg^{+}IgE^{hi}$ group individuals

None of the individuals in the $egg^{+}IgE^{hi}$ group produced detectable levels of IFNγ in response to HIV-1 p24 stimulation. The group median differed significantly from the $egg^{+}IgE^{lo}$ group (p=0.01), from the $egg^{-}IgE^{hi}$ group (p=0.04) and from the $egg^{-}IgE^{lo}$ group (p=0.05).

IL-2 expression in response to HIV-1 p24 stimulation was generally low in all groups however; none of the PBMCs obtained from the $egg^{+}IgE^{hi}$ group participants seemed to produce it upon challenge with HIV-1 p24 (Table 5.3). This was similar to the findings in unstimulated cytokine results wherein these individuals had undetectable levels of IL-2.

5.3.9.4 The egg^{+}IgE^{lo} group had higher IFNγ responses to HIV-1 p24

The highest median IFNγ of 11.1pg/ml (Table 5.3) was found in the egg^{+}IgE^{lo} group and this was significantly higher than the value for the egg^{+}IgE^{hi} group (p=0.02).

The regulatory cytokine IL-10 median was three- to four- folds higher in the egg^{+}IgE^{lo} group compared to the three other subgroups (Table 5.3).

No other distinct patterns were observed in terms of cytokine profiles and HIV/helminth infections in response to HIV-1 p24 stimulation.

Table 5. 3. Cytokine production in response to HIV-1 p24 challenge.
Expression of cytokines in HIV-1p24 (p24) antigen-stimulated cell culture supernatants in HIV infected groups with or without helminth co-infection. PBMCs at a concentration of $1x10^6$ /ml of cRPMI were stimulated with p24 and cultured at 37°C for five days and supernatants were collected, centrifuged and stored at -70°. Later the supernatants were analysed by the multiplex technology Bioplex (BioRad) for cytokine expression. Results are presented as median, minimum and maximum values for each subgroup.

Cytokine(pg/ml)	Group	Egg^+IgE^{hi}	Egg^+IgE^{lo}	Egg^-IgE^{hi}	Egg^-IgE^{lo}
Proinflammatory					
IL-6 p24	**Median**	**46.47**	**866.2**	**572.9**	**0.0**
	Min	0.00	0.0	0.0	0.0
	Max	1410.2	4599.0	12483	1234.0
IL-8 p24	**Median**	**864.4**	**1032.4**	**591.1**	**0.0**
	Min	0.00	0.0	0.0	0.0
	Max	5292.5	3388.2	3690	1076.0
TNF-a p24	**Median**	**19.97**	**7.0**	**30.44**	**2.89**
	Min	0.00	0.0	0.0	0.0
	Max	249.5	53.3	1380	217.44
Anti-inflammatory					
IL-1RA p24	**Median**	**5.07**	**21.6**	**0.0**	**10.82**
	Min	0.00	0.0	0.0	0.0
	Max	217.8	516	412	317.78
Th_1					
IL-2p24	**Median**	**0.00**	**0.40**	**0.0**	**0.0**
	Min	0.00	0.0	0.0	0.0
	Max	0.000	2.11	3.12	4.55
IFN-γ p24	**Median**	**0.00**	**11.1**	**1.90**	**1.38**
	Min	0.00	-0.38	-0.39	0.0
	Max	0.000	65.9	497.2	63.13
IP-10 p24	**Median**	**9.25**	**23.5**	**40.18**	**78.86**
	Min	0.00	0.0	0.0	0.0
	Max	104.3	101.2	1708	874.98
GM-CSFp24	**Median**	**0.00**	**13.5**	**8.51**	**0.0**
	Min	0.00	0.0	0.0	0.0
	Max	96.380	65.2	226.8	95.47
Th_2					
IL-4 p24	**Median**	**0.00**	**0.0**	**0.0**	**0.0**
	Min	0.00	0.0	0.0	0.0
	Max	4.680	4.8	7.04	2.25
IL-5 p24	**Median**	**0.00**	**0.0**	**0.0**	**0.0**
	Min	0.00	0.0	0.0	0.0
	Max	0.03	1.11	1.75	0.08
IL-13 p24	**Median**	**0.00**	**2.25**	**0.0**	**0.0**
	Min	0.00	0.0	0.0	0.0
	Max	2.24	45.22	11.4	2.24
Regulatory					
IL-10 p24	**Median**	**10.23**	**33.9**	**8.69**	**11.19**
	Min	0.00	0.0	0.0	0.0
	Max	109.6	94.6	305.3	104.9

*Eggs- faecal helminth eggs. Hi / lo IgE- high and low serum *Ascaris lumbricoides*-specific IgE. IL interleukin; IL-1RA interleukin-1 receptor antagonist; IFNγ interferon gamma; IP-10 interferon-inducible protein 10; TNFα Tumour necrosis factor alpha; GM-CSF granulocyte-monocyte stimulation factor; RPMI=complete RPMI medium.

5.3.10 Cytokine production by Ascaris worm-challenged cells

Although antigen specific responses to challenge with worm antigen generally invoked stronger responses compared to HIV antigen stimulation assays (Tables 5.3 and 5.4), levels of the anti-inflammatory cytokine IL-1RA and most IP-10 levels were undetectable in these assays. As in HIV-1 p-24 stimulated assays, no distinctive patterns of cytokine profiles were discernible in terms of interactions between helminth and HIV infections in the worm antigen stimulation experiments.

5.3.10.1 The egg^{+}IgE^{hi} and egg^{-I}gE^{lo} subgroups

Comparison between the egg^{+}IgE^{hi} and Egg^{+}IgE^{lo} subgroups showed that all the pro-inflammatory cytokines were higher but not significant in the former. No significant differences were observed between the two groups for the Th_1, Th_2 and regulatory cytokines in Ascaris-stimulated cultures.

5.3.10.2 The egg^{+}IgE^{lo} subgroups has high proinflammatory IL-6 and IL-8

The one outstanding finding was the significantly higher levels of the proinflammatory cytokines IL-6 and IL-8 found in the egg^{+}IgE^{lo} group (p=0.03 and p=0.04 respectively, compared to the egg^{-}IgE^{lo} subgroup..

Table 5.4 Group medians for cytokine responses to Ascaris crude antigen stimulation.

Median percentage expression of extracellular cytokines in *Ascaris* antigen-stimulated cell cultures in HIV infected groups with or without helminth co-infection were determined. PBMCs at a concentration of $1x10^6$ per milliliter of cRPMI were stimulated and cultured at 37°C for six days and supernatants were collected, centrifuged and stored at -70°. Later the supernatants were analysed by the multiplex technology Bioplex (BioRad) for cytokine expression.

Cytokine(pg/ml)	Group	Egg$^+$IgE^{hi} N=10	Egg$^+$IgE^{lo} N=10	Egg$^-$IgE^{hi} N=10	Eggs$^-$IgE^{lo} N=9
Proinflammatory					
IL-6 Asc	**Median**	**637.4600**	**2086.665**	**631.9650**	**318.2400**
	Min	0.0	0.0	0.0	0.0
	Max	1756.83	6513.45	12877.72	1919.01
IL-8 Asc	**Median**	**230.5550**	**1250.360**	**31.0250**	**0.0000**
	Min	0.0	0.0	0.0	0.0
	Max	4602.59	4112.02	3619.98	959.93
TNF-a Asc	**Median**	**123.0000**	**60.880**	**65.5550**	**28.8500**
	Min	0.0	0.0	10.48	0.0
	Max	219.24	218.9	1510.97	388.6
Th$_1$					
IL-2 Asc	**Median**	**0.5650**	**1.655**	**2.8200**	**0.4200**
	Min	0.0	0.0	0.0	0.0
	Max	4.43	5.51	8.17	164.46
IFN-g Asc	**Median**	**0.000**	**1.810**	**14.0350**	**0.2200**
	Min	0.00	0.0	0.0	0.0
	Max	13.750	98.66	600.41	110.11
IP-10 Asc	**Median**	**0.0000**	**0.000**	**0.0000**	**0.0000**
	Min	0.00	0.0	0.0	**0.0**
	Max	86.540	4.63	153.79	**119.17**
GM-CSF Asc	**Median**	**15.6950**	**32.640**	**37.7850**	**10.8200**
	Min	0.0	0.0	0.0	0.0
	Max	217.84	516.71	452.38	325.47
Th$_2$					
IL-4 Asc)	**Median**	**0.0000**	**4.440**	**6.5500**	**2.1000**
	Min	0.0	0.0	0.0	0.0
	Max	8.39	12.23	102.91	26.23
IL-5 Asc	**Median**	**0.2100**	**0.240**	**4.6650**	**0.0100**
	Min	0.0	0.0	0.0	0.0
	Max	3.84	22.65	459.83	95.79
IL-13 Asc	**Median**	**5.5250**	**18.095**	**80.5650**	**2.3700**
	Min	0.00	2.47	0.0	0.0
	Max	28.130	355.05	2120.11	1903.42
Regulatory					
IL-10 Asc	**Median**	**60.9500**	**76.900**	**52.3250**	**33.2600**
	Min	0.00	0.0	0.0	0.0
	Max	138.090	302.5	323.83	247.49

*Eggs- faecal helminth eggs. Hi IgE- high serum *Ascaris lumbricoides*-specific IgE. IL interleukin; IL-1RA interleukin-1 receptor antagonist; IFNγ interferon gamma;IP-10 interferon-inducible protein 10; TNFα Tumor necrosis factor alpha; GM-CSF granulocyte-monocyte stimulation factor; cRPMI=complete RPMI medium. Preparation of the Ascaris crude antigen detailed in Appendix 6. page 254..

5. 4 Summary findings

There were some assay limitations in the present chapter, however some distinctions were revealed within the groups who differ in terms of proliferative responses, in constitutive and in stimulated cytokine production. The first is the $egg^{+}IgE^{hi}$ group: When compared with the HIV singly infected $egg^{-}IgE^{lo}$, some differences were noted in this dually infected subgroup.This group was shown to have high background expression of Ki67 and CTLA4. Antigen specific proliferation tended to be lower despite potent mitogen responses in this group. Cytokine profiling showed that at baseline as well as in response to HIV-1 p24 challenge this group persistently had low and/or undetectable Th_1 cytokines (IL-2, IFNγ and IP-10).

The second distinction was that high IgE responder groups (the $egg^{+}IgE^{hi}$ and the $egg^{-}IgE^{hi}$) typically had higher background IL-4 (Th_2) and lower Th_1 cytokines (IL-2 and IP-10) with lower IL-10 levels.

Finally the $egg^{+}IgE^{lo}$ group had higher levels of Th_1 cytokines in unstimulated and HIV-1 p24 stimulated cell responsesas well as lower background IL-4 (Th_2). IL-10 levels were high at baseline, in HIV-1 p24- and Ascaris worm-challenged cells in this group. Remarkably higher levels in IL-6 and IL-8 were also noted in this subgroup in ascaris stimulated cells.

5.5 Discussion

According to the study hypothesis, worm infection leads to predominance of type 2 cytokines, or conversely, diminished type 1 responses. Further, expression of proinflammatory cytokines is suggested to increase during worm infection and as HIV infection progresses (Kedzierska and Crowse, 2001; Lawn *et al.*, 2001). Increases in type 2 and inflammatory cytokines is associated with HIV disease progression (Kedzierska and Crowse, 2001; Borkow and Bentwich, 2004) while the proinflammatory cytokine TNFα is reported to enhance HIV replication (Lawn et al. 2001). With regards to HIV and worm co-infection, this would impair effective HIV immune control and accelerate progression to AIDS. Furthermore, the proliferative capacity of cells from individuals with HIV (Day *et al.*, 2007) or both HIV and helminth infections (Borkow and Bentwich, 2004) is attenuated with a deleterious effect on immune competence. Thus, to further investigate whether individuals with both infections are immunologically compromised in terms of HIV immunity, the proliferative capacity and cytokine profiles were analysed in unstimulated and antigen-stimulated PBMC cultures.

Ki67 is well recognised as a proliferation-related nuclear protein (Gerdes, 1990). Its expression is therefore used as a marker for proliferating cells and was used in this work to characterize the capacity of PBMCs to proliferate in response to antigenic stimulation.

CTLA-4 protein expression is induced by activation and binds the B-7 molecules with higher affinity than CD28 (the costimulatory molecule). This binding inhibits T cell signaling thereby downregulating the activation process (Janeway *et al.*, 2001). As such the expression of CTL-A4 counteracts immune activation and can be a phenotypic marker of activation.

The results of the present section, notwithstanding the design limitations, tend to suggest that dual infection with HIV and $egg^{+}IgE^{hi}$ status lends support to the study hypothesis.

Firstly, expression of Ki67 (the cell proliferation nuclear protein) was significantly increased in PBMCs of participants in the $egg^{+}IgE^{hi}$ subgroup in the absence of stimulating antigen, This was observed even when this subgroup was compared to the

HIV+ $egg^{-}IgE^{lo}$ single infected individuals.Expression of CTLA-4 molecule was also significantly increased in unstimulated PBMCs of individuals in the $egg^{+}IgE^{hi}$ group. Ki67 is expressed by proliferating cells at G_1, S and M phases of the cell cycle (Iatropoulous and Williams, 1996). Brunner *et al.* (1999) showed that CTLA-4 inhibits cell cycle progression through reduction of cyclin-dependant kinases. In 2001, Leng *et a*l. illustrated that simultaneous increase of both Ki67 and CTLA-4 indicates activated cells arrested at the G_1 phase and therefore not necessarily proliferating cells. Therefore the finding of marked increase of these molecules in unstimulated cells of participants with both HIV and $egg^{+}IgE^{hi}$ helminth infection strongly suggests immune hyperactivation. This corroborates the findings in Chapter 4 of this thesis wherein all activation markers were highly increased in this group.

Secondly, the HIV-1 positive, $egg^{+}IgE^{hi}$ worm co-infected individuals had significantly higher responses to mitogen challenge when compared to the $egg^{-}IgE^{lo}$ group (without helminth infection), implying that their cells were viable and capable of proliferation. However this $egg^{+}IgE^{hi}$ subgroup showed decreased proliferative (Ki67) responses to HIV-1 p24, streptokinase and Ascaris worm antigens (Figure 5.2 PanelB) and typical dotplots in Figure 5.4 although these were not statistically significant. Expression of CTLA-4 in response to these specific antigens was similar in all the subgroups, without any significant differences thus showing that the Ki67 reductions corresponded to decreased proliferation per se.

The lower proliferative responses to specific antigens and strong mitogen responses in the dually infected HIV+ $egg^{+}IgE^{hi}$ individuals agree with previous reports that HIV and helminths selectively target and downregulate specific responses directed at them: HIV tat was shown to block CD26 (a costimulatory molecule) thereby attenuating effective transduction of the stimulation signals (Subramanyam et al., 1993). Likewise, both HIV and helminths are documented to downregulate expression of another costimulatory molecule, CD28 (Borkow and Bentwich, 2004), thus inducing generalised and specific anergy to themselves and other infecting pathogens.

Notably, in this study, there were significantly strong PHA responses in all the subgroups with evidence of helminth exposure (either stool positive and/or high Ascaris IgE) in the HIV infected group, also suggesting that these cells retain their capacity to proliferate in

response to non-specific stimulation. The results then further support the suggestion that antigen specific responses are compromised in the presence of HIV- helminth co-infections.

The second part of this chapter examined the cytokine responses with and without antigen specific challenge in HIV-1 infected subgroups only. Unstimulated PBMC cytokine assays presumably represent the ex vivo profile of background cytokine production with a reasonable measure of inaccuracy. In the case of HIV and worm co-infections this may represent cytokine production in response to the two infecting pathogens. Upon analysis of unstimulated cytokine production patterns certain findings of the present section were inclined towards the study hypothesis.
When the HIV+ $egg^{+}IgE^{hi}$ subgroup was compared with the HIV singly-infected $egg^{-}IgE^{lo}$ individuals, there were significantly lower levels of the anti-inflammatory cytokine(IL-1RA) p=0.04 and trends for lower type 1 cytokines IL-2(p=0.07) and IFNγ (p=0.09). In HIV p24 stimulated cultures, the Th_1 cytokine IP-10 was also significantly lower in the $egg^{+}IgE^{hi}$ individuals. Within all the HIV positive subgroups there was an association between $egg^{+}IgE^{hi}$ worm infection status and low baseline production of type 1 cytokines. IL-2 and IFNγ were undetectable and significantly lower while IP -10 median was lowest in individuals with HIV and $egg^{+}IgE^{hi}$ worm infections. The significantly lower Th_1 cytokines, more importantly IFNγ and IL-2 in this group has serious implications for HIV disease whose control is largely mediated by these cytokines. IL-2 is a major lymphoproliferative cytokine that also drives differentiation of lymphocytes into armed effector cells while IFNγ, the antiviral cytokine plays a pivotal role in elimination of virally-infected cells (Janeway *et al*., 2001). Production of these two cytokines was lower not only in unstimulated PBMCs but also in cells stimulated with the HIV-1 p24 antigen. Lower levels of IL-2 by the $egg^{+}IgE^{hi}$ group is also in line with the tendency towards low antigen-specific proliferative capacity in these individuals as shown in section 5.3.4.2.
Furthermore, the significant reduction of IL-2 and IFNγ with a tendency towards decreased antigen-specific proliferative responses in the $egg^{+}IgE^{hi}$ group corresponds with the notion that defective IL-2 production is associated with advancing HIV immunodeficiency (Clerici and Shearerr, 1994). In addition, the simultaneous reduction of IL-2 and IFNγ in this group tallys with the findings by Sousa *et al* (2005) confirming that HIV-1 progression is associated with reduction of the population of $CD4^{+}$ lymphocytes with potent proliferative capacity and ability to produce IL-2 and IFNγ.

Indeed these authors demonstrated the recovery of this population after 4 weeks of highly active antiretroviral therapy (HAART). In the same context, the results of the present section corroborate the findings in Chapter 3 of this thesis where a tendency towards reduction of $CD4^+$ lymphocyte numbers in the egg^+IgE^{hi} group was observed, although absolute total numbers, not the functional aspect of CD4+ cells were analysedin this work. In addition, in the first section of this chapter it was shown that CTLA-4 expression was significantly increased in this egg^+IgE^{hi} group. CTLA-4 inhibits production of IL-2 among other downmodulatory functions (Janeway *et al.,* 2001). These provide an immunological basis that is in line with the hypothetical interaction between HIV and helminth coinfection (particularly in the egg^+IgE^{hi} individuals).
These, and the fact that comparison between the egg^+IgE^{hi} and egg^-IgE^{lo} individuals revealed lower trends for background IL-2 and IFNγ and significantly lower anti-inflammatory IL-1RA is further suggestive of an inclination for support of the study hypothesis that dual helminth and HIV infection impairs type 1 cytokine responses that are critical in anti-HIV cell mediated immunity.

Secondly, high IgE responder subgroups (egg^+IgE^{hi} and egg^-IgE^{hi}) had significantly higherbackground IL-4 levels as expected. In addition these two groups also had lowest median values for the type 1 cytokines IL-2 and IP-10. High IgE and IL-4 production are typical Th_2 helminth responses. Their association with reduction of type 1 cytokines, as found in the present work concurs with the hypothesized compromise in type 1 response to HIV as a result of helminth-induced Th_2 biasing.

Thirdly, a noteworthy observation that the stool egg positive groups (egg^+IgE^{hi} and egg^+IgE^{lo}) had higher baseline levels of TNFα may be biologically relevant although it was not statistically significant. Egg excretion may be associated with ongoing inflammatory processes (Secor *et* al., 2003) hence TNFα increased accordingly in the stool egg excretors in this study. This cytokine is reported to stimulate HIV replication (Lawn e*t al.*, 2001). The elevated TNFα by egg excretors supports the suggestion that helminth infection results in increased levels of proinflammatory cytokines, particularly TNFα, and thus hypothetically could accelerate HIV-1 replication.

The background levels of the other inflammatory cytokines IL-6 and IL-8 and the anti-inflammatory IL-1RA were very high such that they were extrapolated outside the upper

range of the standard curve. The interpretation therefore could be inaccurate and will not be discussed in terms of co-infection interactions. It should be noted as well that the expression of IL-6 is dependent on the stage of the infection. It was shown *in vitro* that expression of IL-6 in helminth infection is stage-dependent: acute infections produced low levels while chronically infected cells produced higher levels of IL-6 (Rodriguez-Sosa *et al.*, 2002). The obscure results found in the present work could be explained in part by the fact that the duration of both HIV and helminth infections in all the subgroups examined in this study are not known. Some individuals could be at the chronic stages of infections while others recently acquired the infections just prior to study commencement. The finding that significantly lower levels of IL-8 were accompanied by lower IL-1RA (Table 5.3) in the stool egg positive groups is also at variance with the documented inverse relationship between these cytokines. IL-1RA was reported to decrease IL-1-induced production of IL-6 and IL-8 (Garat and Arend, 2003), and thus is classified as an anti-inflammatory mediator in this context. This discordance further affirms that the interpretation of the profile of IL-6, IL-8 and IL-1RA may not be straight forward in the context of co-infection and need further investigation.

The final section of the results analysed cytokine production after stimulation of PBMCs with HIV-1p24 and *Ascaris* worm antigens. In this section a remarkable observation was the generalised marked reduction in the level of cytokine production in response to the specific antigens compared to those secreted by unstimulated cells. This was particularly evident in the proinflammatory cytokines in HIV-1 p24 stimulation assays, (Table 5.3).

A prominent finding in the HIV-p24 antigen stimulation assays was the significantly lower IFNγ (type1 response) in the egg^{+}IgE^{hi} group when cells were stimulated with HIV-p24 antigen. As discussed above, IFNγ plays an important role in HIV immune control.

In *Ascaris* worm stimulated assays, significantly lower levels of IL-6 and IL-8 were observed in the egg^{-}IgE^{lo} group (Table 5.4), which is in keeping with the fact that these are acute proinflammatory cytokines, which may not be expected to rise in response to worm challenge in PBMCs from helminth uninfected individuals. On the other hand, these two inflammatory cytokines were significantly increased in the egg^{+}IgE^{lo} subgroup. As indicated in Chapter 3, the egg^{+}IgE^{lo} resemble the modified Th$_2$ phenotype. The increased IL-6 and IL-8 found in these egg^{-}IgE^{lo} individuals corresponds well with the

assertions that (i) the modified Th_2 individuals are susceptible to recurrent, heavy helminth infections (Maizels and Yashdanbakhsh, 2003) and (ii) helminth-chronically infected cells produced higher levels of IL-6 (Rodriguez-Sosa *et al.*, 2002).

It was observed that the patterns of many of the antigen-specific (HIV-1 p24 and *Ascaris* worm antigens) cytokine responses were not clearly defined. A combination of factors possibly contributed to this: While *in vitro* antigen stimulation in the presence of added co stimulatory molecules may mimic the antigen processing, presentation and associated signal transduction in the human body, many dynamic normal physiologic factors may be “distorted” during the *in vitro* modeling of the process, and in particular use of PBMCs that had been frozen. For example Godoy-Ramirez *et al* (2004) recommended use of diluted whole blood for optimized antigen specific responses in culture for intracellular cytokine assays. Ideally freshly drawn whole blood, which is superior to previously frozen PBMCs, should have been used in conjunction with intracellular cytokine staining to detect antigen specific responses in the present study. However, the ideal was compromised because of technical logistics and because the same set of PBMCs had to be split and used for surface staining of activation markers and the remainder used for culture. Following culture the cells had to be split for Ki67 staining and other assays. It was a challenge to find individuals with adequate numbers of cells to do all the assays particularly because of the generally low counts in HIV infected participants.

Another limitation of this section of the study was difficulty to find balance for optimum incubation period for all the cytokines analysed, against the requirement for longer incubation to expand antigen-specific cellular responses (Godoy-Ramirez *et al.*, 2004). Although optimisation experiments were undertaken, owing to cost implications, only two cytokines were investigated (IFNγ and IL-4) from which an optimum of five days was found for antigen-specific responses. Furthermore, bulk culture of PBMCs for cytokine assays to define antigen-specific responses may not be a true reflection of the *in vivo* setting, particularly under conditions of HIV and helminth infections: T lymphocytes are mainly the major source of many cytokines and in addition monocytes and macrophages produce some (Janeway *et al.*, 2001). Disruptions in lymphocyte populations as found during HIV and helminth infections, and the redundant nature of cytokine production may therefore confuse the interpretation of such responses in bulk PBMC cultures. In addition, re-stimulation of PBMCs with cognate antigen reactivates the cells which then

enter into cell cycling. This process is regulated by cytokines. The cytokines in turn will regulate Suppressors of Cytokine Signalling (SOCS) at a later stage of activation in order to regulate and maintain homeostasis (Yu *et al.*, 2003). It is expected that the kinetics of this process differs for each cytokine. Stringent control of these cycles for each cytokine in culture would therefore be necessary to obtain accurate optimum secretion and downregulation timepoints, which was not feasible in this work. This may be one of the reasons why some cytokine levels were distinctly lower in specific antigen stimulated than unstimulated cells (the negative feedback cycle could have been activated by antigen stimulation in the 5 day culture).

Finally, culture of PBMC *in vitro* for five days may have increased the apoptosis for two reasons. *In vitro* assays of PBMCs are known to be more likely to increase antigen-induced apoptosis while also, cells of HIV-infected individuals are documented to be highly susceptible to programmed cell death (Minami *et al.*, 2006; Porichis *et al.*, 2007). As a result determination of cytokine levels under these conditions may affect some or all of the results. Some or all of the factors discussed above may have contributed to the limited findings in antigen-specific cytokine responses in the present work.

Another major finding of this study is the subset of individuals who were excreting eggs but have low *Ascaris* IgE, defined as $egg^{+}IgE^{lo}$. This group had higher background levels of type 1 cytokines IFNγ, IL-2 and IP-10 plus the lowest IL-4 levels. Even in HIV-1 p24 stimulated assays this group had a higher IFNγ response. Of note, the levels of the regulatory cytokine IL-10 were highest at baseline, in HIV-1 p24 and worm antigen stimulated cells in these individuals. IL-10 downregulates antibody class switching to IgE (Maizels and Yazdanbakhsh, 2003). Production of this antibody is promoted by IL-4 (Janeway *et al.*, 2001: 370). Therefore the profile of $egg^{+}IgE^{lo}$ group fits perfectly with the modified Th_2 model proposed by Maizels and Yazdanbakhsh (2003). These authors described three types of Th phenotype outcomes that follow after exposure to helminth- (*Schistosoma* and *Brugia malayi*) infections. The first, referred to as modified Th_2 phenotype is characterized by clinically asymptomatic helminth infections and the individuals are highly susceptible to persistent heavy infection and constitute the main reservoir for transmission. They have marked regulatory cell (T_{reg}) activity accompanied by high IL-10 levels, low IgE and high IgG_4. In the present work, the $egg^{+}IgE^{lo}$ group

typify this modified Th_2 response by consistently displaying high IL-10 levels and low IgE responses in the presence of infection (faecal eggs). In chapter 3 this group was shown to have a relatively competent immune response to HIV; they had higher $CD4^+$ counts, lower viral load and higher proportions of lymphocyte subsets.

A second phenotype described as the uncontrolled Th_1, in which the individuals are susceptible to infections and have uncontrolled inflammatory disease, , high IgE and low IgG_4 subclass. In the present study, this may be represented by the group with egg^+IgE^{hi} worm infection, having high *Ascaris* IgE and excreting faecal eggs. This group (together with the egg^+IgE^{lo} group) had higher median values for TNFα (proinflammatory) which corresponds well with the described phenotype. It is noted that there is an anolmally in terms of HIV response in these individuals. Although they are designated to be uncontrolled $Th_{1,}$ by Maizels and Yazdanbakhsh (2003), in our work they were unable to produce the same Th_1 cytokines against HIV or worms in culture. In the two dimensional immunological map presented by Diaz and Allen (2007), the Th_1–Th_2 axis is superimposed with the inflammatory-regulatory axis, with various types of infections distributed along the four planes, for example the helminths are on the Th_2- regulatory end while allergy and autoimmunity are on the opposing Th_2-inflammatory end of the map. In this model, these authors suggest that this separation along the inflammatory-regulatory axis is determined by both the differences in the strength of the Th17 response as well as the presence of the T_{reg} cells. The Th17 cells are key to the inflammatory component of the model and are known to play a key role in innate immune responses against extracellular bacteria (Kolls and Linden, 2004). Their role in human HIV and helminth infections has not been fully defined. Cecchinato *et al.,* (2008) showed that in the SIV–macaque model, this subset of $CD4^+$ T cells is diminished in the mucosal and systemic sites compared to Th_1 cells. Helminths are documented to suppress the immune system by various mechanisms including alteration of the the innate immune cells such as the macrophages and dendritic cells (Rodriguez-Sosa *et al.* 2002; Maizels *et al.*, 2004). The answers to this inconsistency may possibly lie in the ability of helminths to interefere with immune cell function, specifically the mechanism by which Th17 cells interact with the T_{reg} and the Th_1 cells to produce the phenotype observed in this study .It is likely that the final outcome observed in this group, that is their inability to control worms allows the worms to activate immune responses that are detrimental to the host in controlling HIV through manipulation of the Th_1,Th17 and T_{reg}

balance. In addition, the fact that the classification by Maizel and Yazdanbakhsh in 2003 did not take the role of the inflammatory Th17 cells into consideration may have resulted in a misnomer of the "uncontrolled Th_1" description in our speculative interpretation with the recent model by Diaz and Allen (2007). Another possibility would be the fact that the present work analysed responses to the *Ascaris lumbricoides* and *Trichuris trichuria* while Maizel and Yazdanbakhsh (2003) based their classification on responses to *Schistosoma* and *Brugia malayi*. The differences in the immune responses to the different types of helminths have been suggested (Modjarrad and colleagues, 2005) In the context of the present findings, uncontrolled Th1 activity was not evident.

The third phenotype is characterized by balanced $Th_1/Th_2/T_{reg}$ cell activities with proportional distribution of the IgE and IgG_4 antibody isotypes and regulatory cell activity. The balanced Th_1/Th_2 response is sufficient to kill the invading parasites and hence the group is classically resistant to infection. In our work this phenotype would be represented by the individuals with high IgE but no faecal eggs (the egg^-IgE^{hi} group).

These finding are significant because they reveal an important aspect of classifying helminth infection. It is apparent that the different phenotypes of helminthiasis impact differently on the immune responses to infections including HIV as shown by findings in this study. The value of serological classification in addition to stool egg positivity therefore can not be overemphasized.

5.6 Conclusions

Despite the limitations of the study, some findings emerged which are inclined towards support of the study hypothesis. Stool egg positivity with high Ascaris specific IgE in HIV infected participants consistently showed impaired immune responses: simultaneously elevated Ki67 and CTLA-4 consistent with increased immune activation, a tendency towards antigen specific, proliferative hyporesponsiveness, impaired type 1 cytokine responses in unstimulated and antigen stimulated cells and increased background TNFα levels.

High IgE responders also showed a tendency towards this impairment of type 1 responses while egg excretion was associated with a tendency towards elevated TNFα.

Finally, the egg^+IgE^{lo} group displayed an immune profile that is consistent with the modified Th_2 phenotype.

Taken together, these findings are suggestive of possible support for the hypothesis under investigation, with a prominent interaction between egg^+IgE^{hi} worm infection and HIV co-infection., However the experimental limitations restrict any direct interpretation and inference being made from these findings. Future studies could improve on the shortcomings highlighted in the limitations of the section above.

References.

Actor JK, Shirai M, Kullberg MC, *et al* . 1993. Helminth infection results in decreased virus-specific CD8+ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. *Proceedings of the National Academy of Science of USA*. **90 (3):** 948-953.

Bahbouhi B, Landay A, Al-Harthi L. 2004. Dynamics of cytokine expression in HIV productively infected primary CD4^{+} T cells. *Blood.* **103 (12):**4581-4587.

Bentwich Z, Kalinkovich A, Weisman Z, Borkow G, Beyers N, Beyers AD. (1999). Can eradication of helminth infections change the face of AIDS and tuberculosis*?* *Immunology Today*. **20(1):** 485-487.

Boaz MJ, Waters, A, Murad S. *et al.* 2002. Presence of HIV-1 Gag-specific IFNγ+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with non-progression in HIV-1 infection. *The Journal of Immunology***.169:**6376-6385**.**

Borkow G, Qibin Leng Q, Ziva Weisman Z, *et al.* 2000.Chronic immune activation associated with intestinal helminth infections results in impaired signal transduction and anergy. *Journal of Clinical Investigation.* **106(8):** 1053–1060**.**

Borkow G, Bentwich Z. 2004. Chronic immune activation associated with chronic helminthic and Human Immunodeficiency Virus infections: Role of hyporesponsiveness and anergy. *Clinical Microbiology Reviews.* **17(4):** 1012-1030.

Brown M, Mawa PA, Kaleebu P, Elliott M. 2006. Helminths and HIV infection: epidemiological observations on immunological hypotheses. *Parasite Immunology.* **28(11):** 613–623.

Brunner MC, Chambers CA, Chang FK, *et al.* 1999. CTLA-4 mediated inhibition of early events of T cell proliferation. *The Journal of Immunology* **. 15:**5813-5820.

Carrington M, Nelson G, O'Brien SJ. 2001. Considering genetic profiles in functional studies of immune responsiveness. *Immunology Letters.***79:**131-140.

Cecchinato V, Trindade CJ, Laurence A, *et al.* 2008. Altered balance between Th17 and Th1 cells at mucosal sites predicts AIDS progression in simian immunodeficiency virus-infected macaques. *Mucosal Immunology* **1**: 279–288.

Clerici M, Shearer GM. 1994. The Th_1 -Th_2 hypothesis of HIV infection: new insights. *Immunology Today*. **15 (12):**575-581.

Cooper PJ, Espinel I, Parades W, *et al.* 1998. Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocercariasis : a possible role for interluekin-10. *The Journal of Infectious Diseases*. **178 (4):**1133-1138.

Cooper PJ, Chico ME, Losonsky G, *et al.* 2000. Albendazole treatment of children with ascariasis enhances the vibriocidal antibody response to the live attenuated oral cholera vaccine CVD 103-HgR. *The Journal of Infectious Diseases***. 182.** 1199-1206**.**

Cooper PJ, Chico ME, Sandoval C, *et al.* 2001. Human infection with *Ascaris lumbricoides* is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR*. Infection and Immunity*. **69(3):** 1574-1580.

Day CL, Kiepiela P, Leslie AJ, *et al.*, 2007. Proliferative capacity of epitope-specific CD8 T-cell responses is inversely related to viral load in chronic Human Immunodeficiency Virus type 1 infection. *Journal of Virology***. 81 (1):** 434-438.

Diaz A, Allen JE. 2007. Mapping immune response profiles: The emerging scenario from helminth immunology***.*** *European Journal of Immunology*. **37:**1-8.

Elias D, Wolday D, Akuffo H,*et al.*, 2001.Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before abnd after bacille-Calmette-Guerin (BCG) vaccination. *Clinical Experimantal Immunology.* **123:**219-225.

Garat C, Arend WP. 2003. Intracellular IL-1Ra type 1 inhibits IL-1-induced IL-6 and IL-8 production in Caco-2 intestinal epithelial cells through inhibition of p38 mitogen-activated protein kinase and NF-κβ pathways. *Cytokines***. 23 (1-2) :** 31-40.

Gerdes J. 1990. Ki-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. *Seminars in Cancer Biology.* **1 (3) :**199-206.

Godoy-Ramirez K**,** Franck K, Mahdavifar S, *et al.* 2004. Optimum culture conditions for specific and nonspecific activation of whole blood and PBMC for intracellular cytokine assessment by flow cytometry. *Journal of Immunological Methods***. 292 :**1-15.

Grogan JL, Kremsner PG, Deelder AM, Yazdanbakhsh M. 1998. Antigen-specific proliferation and intereferon γ and interleukin-5 production are downregulated during *Schistosoma haematobium* infection. *The Journal of Infectious Diseases***. 177.**1433-1437.

Hazernberg MD, Otto SA, van Benthem BH, *et al*., 2003. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS*. **17:**1881-1888**.**

Iatropoulos MJ, Williams GM.1996. Proliferation markers. *Experimental and Toxicologic Pathology.* **48 (2-3):** 175-181.

Janeway CA, Travers P, Walport M, Shlomchik M. 2001***.*** *In:* Schanck D, Austin P, Lawrence E, *et al*. eds. T-cell mediated immunity**.** *Immunobiology . The immune system in health and disease. 5th ed. Chapters 1-14.* Garland Publishing. Taylor and Francis group

Kedzierska K, Crowse SM. 2001. Cytokines and HIV-1: Interactions and clinical implications***.*** *Antiviral Chemistry and Chemotherapy.* **12 (3):**133-150.

King CL, Mahanty S, Kumaraswami V, *et al.*1993. Cytokine control of parasite specific anergy in human lymphatic filariasis. *The Journal of Clinical Investigation***. 92:** 1667-1673.

Klein SA, Dobmeyer JM, Jurgen M. *et al*., 1997. Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1infection using cytoplasmic cytokine detection on single cell level by flow cytometry. *AIDS.* **11 (9):** 1111-1118.

Kolls JK, Linden A. 2004. Interleukin-17 Family Members and Inflammation *Immunity.* **21(4):** 467-476

Lawn SD, Butera S, Folks TM. 2001. The contribution of immune activation to the pathogenesis and transmission of Human Immunodeficiency Virus type1 infection. *Clinical Microbiology Reviews.* **14(4):** *753-777.*

Leng Q, Borkow G, Weisman Z, *et al*., 2001. Immune activation correlates better than HIV plasma viral load with CD4 T cell decline during HIV infection. *Journal of Acquired Immune Deficiency Syndromes.* **27:** 389-397**.**

Liu Z, Liu Q, Pesce J, *et al.* 2002. *Nippostrongylus* brasiliensis can induce B7-independent antigen-specific development of IL-4-producing T cells from naïve CD4 T cells *in vivo.The Journal of Immunology***.169:**6959-6968

Maizels RM, Balic A, Gomez-Escobar N, *et al*. 2004. Helminth parasites- masters of regulation*. Immunological Reviews***. 201.** 89-116.

Maizels R, Yazdanbakhsh M, 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature Reviews Immunology***. 3:**733-744.

Malhotra I, Mungai P, Wamachi A, et al. 1999. Helminth and Bacillus Calmette-Guérin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *The Journal of Immunology.* **162**. 6843-6848.

Modjarrad K, Zulu I, Redden DT, *et al.* 2005. Treatment of intestinal helminths does not reduce plasma concentrations of HIV-1 RNA in coinfected Zambian adults. *The Journal of Infectious Diseases.* **192:**1277-1283.

Palmer BE, Boritz E, Blyveis N, Wilson CC. 2002. Discordance between frequency of human immunodeficiency type 1 (HIV-1)-specific γ-interferon-producing CD4 T cells and

HIV-1 specific lymphoproliferation in HIV-1-infectedparticipantswith active viral replication. *Journal of Virology.* **76:** 5295.

Rodgriguez-Sosa M, Satoskar AR, Calderon R, *et al.* 2002. Chronic helminth infection induces alternatively activated macrophages expressing high levels of CCR5 with low IL-12 production and Th2-biasing ability. *Infection and Immunity.* **70 (7):** 3656-3664.

Secor WE, Shah A, Mwinzi PMN, et al., 2003. Increased density of human immunodeficiency virus type 1 coreceptors CCR5 and CXCR4 on the surfaces of $CD4^{+}$ T cells and monocytes of patients with *Schistosoma mansoni* infection. *Infection and Immunity.* **71 (11):** 6668-6671.

Sieg S, Bazdar D, Harding C, Lederman M. 2001. Differential expression of interleukin-2 and γ-interferon in human immunodeficiency virus disease. *Journal of Virology***. 75:** 9983.

Sousa A, Chaves A, Doroana M *et al.*, 1999.Kinetics of the changes of lymphocyte subsets defined by cytokine production at single cell level during highly active antiretroviral therapy in HIV infection. *The Journal of Immunology.* **162 :** 3718-3726**.**

Sousa A, Chaves A, Loureiro A, Victorino R. 2001. Comparison of the frequency of interleukin2-, interferon –γ-, and IL-4-producing T cells in 2 diseases, human immunodeficiency virus types 1 and 2, with distinct clinical outcomes. *Journal of Infectious Diseases* **184 :** 552-559.

Subramanyam M, Gutheil WG, Bachovchin, WW Huber BT. 1993. Mechanism of HIV-1 Tat induced inhibition of antigen-specific T cell responsiveness. *The Journal of Immunology,* **150 (6):** 2544-2553**.**

Valdez H, Lederman M. 1997. Cytokines and cytokine therapies in HIV infection. *AIDS Clinical Review*. **6:** 187-228.

Youkeles LH, Soliman MY, Rosenstreich DL.1991. Use of streptokinase as a recall antigen for lymphocyte proliferation testing in vitro *Journal of Allergy and Clinical Immunology.* **88** (2): 166-171.

Yu, C-R, Mahdi RM, Ebong S, Vistica BP, Gery I, Egwuagu CE. 2003. Suppressor of Cytokine Signaling 3 regulates proliferation and activation of T-helper cells. *The Journal of Biological Chemistry*. **278**: 29752-29759.

Chapter 6

Challenges and limitations of the study.

6.0 Introduction

The current research hypothesis is addressing an area that is still largely controversial because similar studies have reported conflicting outcomes, although some reviews have provided strong supportive evidence for immunological interactions between HIV and helminth infection and or treatment, as discussed in the preceeding chapters. For this reason there is a need for properly designed studies that will give conclusive evidence.

Both HIV and helminth infections are chronic. HIV infection has different stages, each characterised by different pathogenesis and immunologic features. Likewise, helminth infections have different life cycle phases, and even feature concomitant immune responses to the various cycles. Thus, an ideal design for studies of co-infections with these two organisms would be a prospective cohort study with randomised sampling. Such a study should also enrol a large enough sample population to allow for statistical power, with participants followed over a substantial time period, in order to derive convincing and conclusive results.

Ideally these studies should be done on both children and pre-teenagers where both worm burdens and viral load tend to be higher, as well as adults. The cohort should then comprise of age and sex matched groups of uninfected, singly and dually infected individuals. For helminths, the groups would need to be stratified for the species as well as worm burdens. The follow up would be determined by the species and each stratified group would be measured for time-to logarithmic CD4 decline or increase in viral load above baseline.

The effects of disease stage and duration would be monitored from the younger generation (presumably the period between non –sexual activity into the period after

approximately one year after sexual activity initiation (for risk of HIV acquisition (ages between 9 and twelve to fifteen). This would enable monitoring of acquisition of helminth infection and HIV seroconversion by regular screening for both infections using both antigen detection (including PCR) and serological markers. The additional requirement for rigorous methods of follow up, regular screening and large sample size would be the additional sampling features necessary for this cohort. Exposures would be measured by demographic profiling,, personal hygiene habits, occupational exposure, socio-economic status (for risk of both infections) as well as sexual risk behaviours. Outcomes would be acquisition of both infections, and also HIV disease progression markers in those infected. These would need to be matched for baseline CD4+ counts and viral loads as major outcomes of disease stage among the HIV singly-or dually infected, and CD4+ counts among the uninfected. The timing of acquisition of the two infections should enable the analysis of bi-directional interactions of the two infections.

The study requirements above dictate the need for substantial funding, more so as it includes costly laboratory assays as well as the costs of maintaining a large number of individuals for follow-up over a long period of time. Being cognizant of the above facts, the current study had a number of challenges which bore inherent limitations. Although these have been highlighted in the relevant chapters in detail, they are summarized in this section.

6.1 Study Challenges

6.1.1 Funding

The first challenge for the present study was to obtain the appropriate funding to meet the study requirements. The budgetary constraints then impacted on the overall study design and methodology limitations as outlined in the following paragraphs. Some of the limitations resulted from logistical issues. These limitations have been alluded to in the relevant chapters; therefore the present section briefly outlines each of them.

6.1.2 Recruitment of HIV negative individuals

One of the criteria for participation in the study was the requirement for HIV testing. At the time of the study's initiation, there were no Antiretroviral Therapy (ART) programmes in public health facilities and it was difficult to motivate people to undergo testing in the absence of available treatment and with the stigmatization that accompanies HIV infection.. As a result the sample sizes for the HIV negative controls became smaller within the subgroups.

6.1.3 Logistics of Laboratory Sample Processing

The ideal was immediate processing of samples as soon as possible after collection. Large numbers of blood samples were drawn on the same day during each visit. Several of the laboratory tests require immediate processing soon after blood is drawn. Although some were processed by the collaborating laboratories (full blood counts, viral load, total and specific IgE) all the immunology assays including all flow cytometry work as well as PBMC separation had to be done by the investigator of this study. Therefore the PBMC had to be isolated on the same day of collection and frozen for later analysis as other high priority analyses had to be undertaken on the same day of collection. Ideally ex vivo PBMC should be analysed soon after separation. In addition, intracellular cytokine staining of whole blood would have been the best choice; however, this assay also requires immediate processing. This would not have been feasible.

6.1.4 The possible presence of undiagnosed chronic infections

The concurrent presence of STIs or latent TB infections could artificially alter the immunological parameters such as the levels of inflammatory cytokines, Th_1-Th_2 cytokine biasing, increased $CD25^+$ regulatory T cell expression and immune activation profiles. This would then affect the overall interpretation of the results in the subgroups. Attempts were therefore made to identify the presence of other infectious diseases such as sexually transmitted infections, tuberculosis and any chronic infections from case record files and through interviewing patients. However, the absolute exclusion of other occult infections may not be guaranteed. If present, and if these had a significant influence on the immune system, these may have affected the results. However it is envisaged that the attempts made to exclude these may have minimised this possible confounder.

6.2 Study Limitations

6.2.1 Study design

A cross sectional design to study the two chronic infections is a major limitation of this work. Although the dates of the first HIV diagnosis were collected from the case record files, this did not necessarily indicate the actual date of seroconversion. Likewise, data on the history or recall of past worm infection was collected, but this also cannot be expected to provide accurate information on worm infections, including chronicity. In all likelihood individuals at different stages of the two infections were analysed together. However, as a pilot study for hypothesis testing, the study sufficed to identify important parameters for designing future research of co-infection as well as providing valuable information that has not been previously reported in South Africa.

6.2.2 Sample size and sampling

Although the sample size was calculated appropriately for power prior to the initiation of the main study, the actual numbers eligible for additional immunological work in this study became smaller. In addition the costs of the laboratory tests did not allow for testing of all available samples.

Sampling bias could also have been introduced during the recruitment of HIV positive individuals who were enrolled to the HIV Support group. Sampling bias in this case refers mostly to the HIV positive group. Owing to the fact that the HIV positive individuals were recruited from attendees of an HIV Positive Support Group, which then limited proper randomisation of these participants. These individuals had already been tested for HIV, some from a previous antenatal screening, although others had tested voluntarily in the local Voluntary Counselling and Testing Facility. Some of the individuals therefore may have (some time before the study commencement), had a clinical condition that made the clinic referral to a VCT facility, although such individuals, and those who had been pregnant in the previous six months, would not have been enrolled into the study (by exclusion criteria).

6.2.3 Lack of adequate numbers of HIV negative controls

To exclude the confounding effects of HIV infection, there should have been similar numbers of adequately matched negative controls for all the assays. Owing to the smaller number of HIV negative individuals enrolled, and also due to the costs of the cytokine assays, this was not possible.

6.2.4 Lack of age-and sex control

Consequent to the limited numbers of participants eligible for all analyses, the ideal comparison method of controlling for age and sex confounding was an additional limitation. Of importance is the fact that both age and sex affect many immunological and haematological parameters.

The majority of participants were females in both HIV positive and negative groups. The HIV negative participants were slightly older (median ages were 30.6 and 40 for the HIV positive and negative groups respectively). These factors are acknowledged as possible contributors to the limitations of the study.

6.2.5 Types of specimens

For cytokine analysis, as mentioned above, more relevant information is obtained from intracellular cytokine staining for specific antigen responses. Use of PBMCs that had been frozen and thawed yields results that are inferior to those obtained from fresh whole blood cultures. However due to logistical problems the PBMCs were the only feasible sample type for additional immunological tests.

Culture of PBMCs from HIV positive individuals for five days further compromised the quality of the results. These cells are documented to be susceptible to increased apoptosis. However, the assay included 13 cytokines, and optimisation assays found five days to be an optimal compromise. The limited number of cells from HIV positive individuals did not allow for a staged collection of supernatants in numerous wells and multiple plates. The most reliable results may be those of the unstimulated cells.

In addition, the use of the dual-platform method for obtaining absolute lymphocyte counts may have had two inherent sources of random laboratory errors. However it is expected that these were minimal as quality control measures were taken during each of the two analyses.

6.2.6 Cohort heterogeneity

The main variability could be the fact that the individuals in the presesent cross sectional analysis cpouild be at different stages of both HIV and helminth infections, while these are chronic manifestations, particularly HIV being a progressive infection.
The inter- and intra- individual variation in many variables in the present study could also constitute a limitation to the study.

Inter individual Variability

The clinical course and outcome of HIV infection is known to vary widely from individual to individual. Such variability includes the differences in susceptibility, viral set point, in the rate of progression to full blown AIDS and the different opportunistic infections manifestations. Amongst the host of factors that are responsible for these variations is the existence of genetic variants (Carrington *et al.*, 2001). Even in helminth infection, differences in worm burden have been associated with genetic predisposition (Quintell, 2003). Several other factors contribute to such variation in the pathogenesis of infections, and these include *inter alia,* environmental factors (hygiene), nutritional status, social behaviour including alcohol consumption, and smoking. Also, although many biological parameters have a normal Gaussian distribution, there is great variation among these both in health and in disease, hence in our study, this variation could also contribute to some bias.

Intra-individual Variation

Variations in some of the outcome variables in the present study, such as CD4 counts (Raboud, *et al.*, 1995) and viral loads (Napravnik *et al.*,2002) have been documented within the same individuals.

It was encouraging to find some significant results in the present work despite the limitations and challenges. These may suggest that the interactions found in this work are considerable enough.

It however is noted that the conclusions derived may have been influenced by the cross sectional design of the study and sampling strategy for the HIV positive group that was attending the support group. If for example the enrolled participants were at an advanced stage of HIV / with acute or chronic helminth infection, the effect of the interaction would be overestimated. On the other hand, if most participants were at an early phase of HIV with acute or chronic helminthiasis, the effect of the relationship between the two infections would be underestimated.

References

Carrington M, Nelson G, O'Brien SJ. 2001. Considering genetic profiles in functional studies of immune responsiveness. *Immunology Letters.* **79:**131-140.

Napravnik S, Poole C, Thomas JC, Eron JJ jr. 2002. Gender difference in HIV RNA levels: A meta-analysis of published studies. *Journal of Acquired Immune Deficiency Syndromes.* **31:**11-19.

Quintell RJ. 2003. Genetics of susceptibility to human helminth infection. *International Journal of Parasitology.* **33:**1219-1231.

Raboud JM, Haley L, Montaner JS, *et al*. 1995. Quantification of the variation due to laboratory and physiologic sources in CD4 lymphocyte counts of clinically stable HIV-infected individuals. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology.* **10:**(Suppl 2):S67-S73.

CHAPTER 7

Discussion and Conclusions

7.0 Overview

The coincident geographic distribution of HIV and helminths in developing countries has led to many immunological hypotheses that suggest a detrimental influence of helminths on HIV pathogenesis. I*n vitro* and *ex vivo* models have been used in studies that attempt to assess this relationship. These have focused mainly on the chronic immune activation that results from prolonged exposure to helminth antigens, the generation of a strong Th_2-biased response and induction of generalised anergy in infested hosts (Lawn *et al*., 2001; Borkow and Bentwich, 2004). Several studies regarding this interaction have been published. They address different aspects of immunological mechanisms of helminth-induced alterations in HIV disease patterns. These include increased susceptibility to the virus (Shapira-Nahor *et al*.,1998; Gopinath *et al*., 2000), augmented transmission through increased expression of receptor molecules (Clerici *et al*., 2000; Kalinkovinch *et al*., 2001; Secor *et al*., 2001), activation, Th_2 -biasing and enhanced replication (Maggi *et al*., 1994; Gopinath *et al.,* 2000; Lawn *et al*., 2001), impaired virus clearance (Actor *et al*., 1993) and accelerated progression to AIDS.

The effects of deworming on HIV progression have also been analysed (Wolday et *al*., 2002; Elliot *et al*., 2003; Lawn *et al.,* 2000; Brown *et al*., 2004; Kallestrup *et al.,* 2005; Modjarrad *et al.,* 2005). Some have reported a beneficial effect of deworming (Wolday *et al*., 2002; Kallestrup *et al.,* 2005) while others have not found this effect. The reasons for the conflicting results are many and varied. Study and experimental designs differed. Some of these studies analysed different species of helminths and it is reported that immunological interaction with HIV is species-specific (Modjarrad *et al.,* 2005). The timing of deworming and analysis of the effects thereof is also suggested to be critical (Brown *et al*., 2006). One of the most important factors was the fact that these studies relied on the presence of eggs in stools to diagnose intestinal nematode infections, although some complemented the diagnosis of schistosomiasis, strongyloides and filariasis with circulating anodic antigen, Elisa, charcoal culture and Knotts concentration, respectively. Use of single stool specimens and microscopy has limited diagnostic value.

Subsequently the risk of misclassifying intestinal helminthiasis is high and this error could have serious implications for studies that purport to determine the effects of co-infection.

In this thesis, two stool samples collected on two consecutive days were used to detect the presence of parasite eggs by the formol ether concentration and Kato-Katz methods. The effect of the two species, *Ascaris lumbricoides* (*Ascaris*) and *Trichuris trichiura* (Tt), on immune responses to HIV was assessed in the present analysis. Indeed, previous work in the study community revealed that these intestinal parasites were the most prevalent. (Anonymous (KTT), 2001, Adams *et al.,* 2006). An important aspect that underpinned this work was the use of serology: *Ascaris lumbricoides*-specific IgE (subsequently referred to as IgE) in addition to stool egg microscopy, to improve the diagnostic sensitivity of the latter in classifying helminth infection status. Use of this serological marker was chosen because at the time, no *Trichuris trichiura* crude antigen was available. However, because of documented extensive antigenic cross reactivity due to antigen sharing between the *Trichuris trichiura* and *Ascaris lumbricoides* species (Lilly-white *et al*., 1991; Bhattacharyya *et al*., 2001), the Ascaris-specific IgE would thus suffice as a serodiagnostic marker for exposure to these two intestinal helminths. To that effect, four subgroups of participants were stratified on the basis of stool egg-positivity /negativity and *Ascaris lumbricoides*-specific IgE levels. These subgroups were the (i) stool egg positive and high IgE ($egg^{+}IgE^{hi}$); (ii) the stool egg positive and low IgE ($egg^{+}IgE^{lo}$); (iii) the stool egg negative and high IgE ($egg^{-}IgE^{hi}$) as well as (iv) the egg negative, IgE low ($egg^{-}IgE^{lo}$) subgroups. The latter status presumably suggests lack of recent infection and/or past exposure to these helminth parasites.

The classification by stool eggs and IgE status proved to be critically relevant because it delineated distinctive groups who proved to be phenotypically different in terms of immunological interactions between helminth and HIV co-infections. The first group presented a typical T-helper 2 (Th_2) immune response to helminths that was consistently detrimental to HIV immune responses, the $egg^{+}IgE^{hi}$ group. A second distinctive phenotype, the stool egg positive and low IgE ($egg^{+}IgE^{lo}$) subgroup of individuals whose response to helminthiasis is consistent with a modified Th_2 phenotype, appears to retain relative immunological competence to control HIV infection. Other patterns of immunological interactions also emerged on the basis of different aspects of helminth

infection. For example some immunological associations (as will be discussed in the following paragraphs) were found in groups with the presence of stool eggs irrespective of IgE levels (egg^+IgE^{hi} and egg^+IgE^{lo}). These include association between stool egg positivity and higher expression of $CD4^+$ CCR5 and $CD8^+CD38^+$ and higher TNFα levels. Other interactions occurred in groups with elevated IgE irrespective of presence or absence of stool eggs (egg^+IgE^{hi} and egg^-IgE^{hi}), such as higher viral loads, lower type 1 cytokines and significant inverse correlation between $CD8^+CD38^+$ and $CD4^+$ counts. These findings are consistent with the model presented by Maizels and Yazdanbakhsh (2003) where they described three phenotypic outcomes of helminth infections, one of which was the modified Th_2 group. These individuals are highly susceptible to helminthiasis but respond with a low IgE, high IgG_4 and high regulatory activity phenotype. The second group responds with a typical high IgE/low IgG_4, uncontrolled Th_1 activity while the third group is charaterised by a balanced $Th_1/Th_2/T_{reg}$ which allow the individuals to clear the infecting parasites.

7.1 The profile of the study participants

Analysis of the general characteristics of the study population in Chapter 2 showed that the prevalence of intestinal helminth infections is high (40-60%) in adults residing in a resource-limited area (Khayelitsha, Western Cape). Estimation of prevalence was improved by use of both egg excretion and serum Ascaris-specific IgE. Furthermore, these individuals presented a high total IgE responder profile (both HIV^+ and HIV^-) as shown by an eight-fold median total IgE above the method reference range in HIV uninfected individuals. Even in the presence of HIV, a five-fold higher level of total IgE was found. This finding concurs with earlier suggestions that Africans generally present with an elevated IgE phenotype and this was concluded from a study conducted in a similar (Xhosa) ethnic group (Gerrard, 1985; Haus *et al.*, 1988). IgE class switching is mediated by $CD4^+$ Th_2 cells, therefore by inference this could suggest a general increase in Th_2 cell populations in these individuals.

Different forms of immune dysregulation by helminths and their effect on HIV responses were assessed within the defined subgroups in Chapters three to five as discussed below.

7.2 Characterisation of T cell subsets, eosinophils and viral loads

In chapter 3, the proposed dysregulation of T cell populations by helminths (Kalinkovinch *et al.*, 1998) and subsequent impaired ability to respond to HIV was assessed through lymphocyte phenotype analysis including total, T, B, NK, $CD4^{+}$ and $CD8^{+}$ lymphocyte counts, eosinophil counts and viral load determination.

It was noted that the expectation based on the study hypothesis was that the HIV singly infected $egg^{-}IgE^{lo}$ group would have a more competent immune profile relative to the HIV+ $egg^{+I}gE^{hi}$ dually infected subgroup.This was however not observed in this section. The two groups had similar numbers of CD8+, NK and B lymphocytes while total lymphocytes and CD4+ cells were lowest in the $egg^{-I}gE^{lo}$ subgroup. Among the HIV negative individuals, some differences were noted between the two groups. Although not statistically significant, all lymphocyte populations were higher in the egg-IgEhi HIV- group compared to the egg+IgEhi subgroup. The smaller sample size in the HIV- group was postulated to influence the powe to detect statistical differences in this group.Under these conditions, a possibility of confounding by HIV was suggested.

.

When the dually-infected subgroups were analysed,several results were consistent with the study hypothesis. Firstly, the $egg^{+}IgE^{hi}$ group had significant eosinophilia, approximately three-fold high viral loads and generally lower absolute counts for all lymphocyte populations. Generalised reduction of lymphocytes may be associated with altered ability to respond to infections including HIV in particular. T lymphocytes play a critical role in containing the virus. Eosinophils are proposed to increase the number of activated cells that are infectable with HIV since they express the receptor CD4 molecule and *in vitro* studies showed that when these cells are activated, they can be infected by HIV (Lucey *et al.*, 1989; Freedman *et al.*, 1991). Their high levels in tissues including vaginal and rectal mucosa could be playing a role in increasing HIV hetero-and homosexual transmission, respectively during helminth infections.

Secondly, the other group with elevated IgE ($egg^{-}IgE^{hi}$ group) also had eosinophilia and three-fold higher median viral load values compared to the low IgE groups (the $egg^{+}IgE^{lo}$ and $egg^{-}IgE^{lo}$ groups). Both eosinophilia and high IgE are classic Th_2 responses that are universally induced by helminth infections. The association of these mediators with

higher viral loads supports the concept that Th_2 polarisation by helminths suppresses the protective Th_1 responses and hence promotes HIV progression (Clerici and Shearer, 2001; Borkow and Bentwich, 2004). A significant negative correlation between viral loads and absolute $CD4^+$ counts was found in all HIV^+ subgroups, but this correlation was strongest in the two high IgE groups. In addition, these groups had the highest proportion of individuals with the highest viral loads. These findings further endorse the notion that Th_2-biased responses are associated with advancing HIV disease (Clerici and Shearer, 2001) with a direct implication for helminths as classic, strong Th_2 inducers and their influence on HIV immune responses.

Lastly,in direct contrast to these responses of high IgE groups, the modified Th_2 responders (egg^+IgE^{lo}) group had significantly higher median absolute $CD4^+$ counts and helper/suppressor ratios, generally higher median absolute numbers of all lymphocytes accompanied by the lowest median viral load. This finding suggests a Th_1 phenotype with a better ability to control the HIV viral infection in these individuals, lending further support to the concept that predominance of a Th_2 response is associated with impaired responses to HIV.

7.3 Immune activation and HIV- helminth co-infections

Immune activation has been widely implicated as playing a pivotal role in HIV pathogenesis through various pathways (Lawn *et al.,* 2001; Borkow and Bentwich, 2004; Kamal and El Sayed Khalifa, 2006). In further addressing the study hypothesis, Chapter 4 sought to define the activation status of the defined subgroups and relate the results to HIV progression. This was achieved through flow cytometric determination of expression of activation markers CCR5, HLA-DR, CD38 and CD71 and correlating these to markers of HIV progression (viral load and $CD4^+$ counts).

Comparison between the dually infected $HIV+egg^+IgE^{hi}$ and the egg^-IgE^{lo} subgroups showed increased expression of activation markers in the dually infected subgroup, which was more than double compared to the levels in the HIV singly-infected (without evidence of helminth infection) egg^-IgE^{lo} group. No significant increase in immune activation was noted in the HIV-singly infected egg^-IgE^{lo} group. CD4+ counts were

similarly low in the two groups (slightly higher, 0.27cells/ml in the egg^{+}IgE^{hi} , compared to 0.22 cells/ml in the egg^{-}IgE^{lo} subgroup).

The findings in this section strongly lend support to the hypothesis: dual infection with HIV and helminths induces immune hyperactivation and impair immune responses to HIV. Firstly, the HIV+, egg^{+}IgE^{hi} group had a highly activated profile as reflected by highly significant increases in median expression of all the measured activation markers (CCR5, HLA-DR, CD25, CD38 and CD71). Nearly all the CD8^{+} cells were CD38-positive in this group. A significant negative correlation between CD4^{+} absolute counts and lymphocyte expression of the classic activation marker (HLA-DR) was found and it was strongest in the egg^{+}IgE^{hi} group. In addition, expression of CD38 on CD8^{+} cells also correlated negatively with CD4^{+} counts and this interaction was higher in this group. The highly activated immune profile found in this egg^{+}IgE^{hi} group concurs with the hypothesis that helminths induce sustained immune activation. The strong inverse correlation between activation and CD4^{+} counts, plus excessive expression of CD38, a poor prognosis marker, strongly suggest progression of HIV infection in this highly immune activated group.Furthermore, the virus burden was higher in this highly activated subgroup, while CD4 counts were. Taken together, these provide strong suggestive evidence that helminth-induced immune activation may be associated with accelerated HIV replication as shown by highest viral load and possibly HIV progression as determined by CD4^{+} decline and CD38 expression.

Furthermore, the notion that HIV replicates better in activated immune cells (Lawn *et al.*, 2001) is strongly supported by these and the finding that this group had the second highest median viral loads (Chapter 3). The concept that helminth infections enhance virus replication in infested hosts through increased immune activation would therefore be compatible with our results. This is supported by the finding that the HIV-singly infected egg^{-}IgE^{lo} subgroup had lower viral load and no increase in immune activation marker expression. The fact that in both HIV+ single infection (egg^{-}IgE^{lo}) and helminth single infection (HIV- egg^{+}IgE^{hi}) there was no remarkable increase in immune activation strongly suggests that dual infection by helminths and HIV increases immune activation which in part or wholly drives virus replication. From our findings it is apparent that this effect is pronounced during a typical helminth (egg^{+}IgE^{hi}) infection.

There were highly significant increased levels of CCR5 expression on $CD4^+$ cells in both groups with positive stool eggs (egg^+IgE^{hi} and egg^+IgE^{lo}). In both these groups there was also significantly high (more than 90%) expression of CD38 on $CD8^+$ cells. Two implications for helminth induced immune dysregulation are interpretable from these findings. Firstly the association between helminth egg-positivity and increased CCR5 expression strongly supports the proposition that helminths are associated with increased susceptibility to, and increased transmission of HIV infection (Borkow and Bentwich, 2004). Increased expression of these two HIV receptor molecules (CD4 and CCR5) increases the number of cells available for new infection as well as expansion of infection to new cells in individuals who are already infected. This finding concurs with those of Secor *et al.* (2001), who found that egg excreting patients with active *Schistosoma mansoni* infection expressed higher levels of the co-receptors CCR5 and CXC4 compared to their treated counterparts. Secondly, high CD38 expression indicates poor prognosis in HIV infection. The relationship between increased expression of this marker and helminth egg excretion lends support to the suggestion that helminth infections accelerate HIV progression.

In addition, the inverse correlation between $CD4^+$ counts and $CD8^+CD38^+$ was strongest in both groups with high IgE (egg^+IgE^{hi} and egg^-IgE^{hi}). This further suggests an association between Th_2 polarisation (high IgE) and advancing HIV disease, as related by declining $CD4^+$ counts and increasing CD38 expression.

A positive correlation existed between viral load and immune activation markers. However this relationship was not as strong as that found between immune activation and $CD4^+$ counts within the subgroups. These results tally with those reported by Leng *et al.* (2001) who described immune activation as correlating better with $CD4^+$ counts than viral load. These results suggest that immune activation has a direct impact on $CD4^+$ cell depletion whereas other mechanisms may affect the impact on virus burden. Indeed other studies have demonstrated that immune activation drives $CD4^+$ depletion (Grossman *et al.*, 2002; Sousa *et al.*, 2002) and is indirectly related to virus burden (Sousa *et al.*, 2002).

7.4 Lymphocyte proliferation and cytokine production

In Chapter 5, the proliferative capacity and cytokine production in response to antigens and mitogen stimulation were evaluated. The Ki67 proliferation antigen and production of thirteen cytokines of different classes (inflammatory, Th_1 and Th_2) were used to investigate *in vitro* cellular immune competence. Peripheral blood mononuclear cells (PBMCs) were cultured for five days unstimulated or stimulated with Phytohaemaglutinin (PHA) mitogen, Streptokinase (recall antigen), recombinant HIV-1 p24 and *Ascaris lumbricoides* crude worm antigens. This approach had a few limitations. First, *in vivo* measurements of cytokine production in response to antigen stimulation are optimally mimicked by antigen-specific intracellular staining of freshly-isolated, diluted whole blood (Godoy-Ramirez *et al.*, 2004). In the present study this ideal could not be achieved for logistical reasons hence the use of bulk PBMCs was resorted to. Secondly, it was challenging to obtain an optimum culture period for a range of thirteen cytokines (although optimisation of conditions was done for IFNγ and IL-4) against the requirement for longer incubation for expansion of antigen-specific responses.

It should thus be noted that the antigen-specific responses for both proliferation and cytokine production studies did not yield very clearly defined patterns of responses. It is highly likely the five-day *in vitro* culture of cells from HIV and helminth infected individuals may have been affected negatively by increased apoptosis. It is reported that the process starts in as few as 12-18 hours in activated cells (Zunino *et al.*, 1996). This could be further complicated by the fact that both HIV and helminth infections are documented to be associated with increased programmed cell death (Borkow and Bentwich, 2004). In addition, Hodge *et al.* (2000) reported that PBMC cultures are more susceptible to apoptosis compared to whole blood. Activation-induced apoptosis in antigen-stimulated PBMC long term cultures of HIV positive individuals could therefore have influenced the results. Most of the significant findings consequently were made on the baseline (unstimulated) assays. Nonetheless we believe that valuable information was obtained from the results of the antigen-stimulated cultures.

In this study, expression of Ki67 as a measure of proliferative potential was complemented by simultaneous measurement of cytotoxic T lymphocyte associated

antigen 4 (CTLA-4) to differentiate between activation- and proliferation-induced elevation of Ki67 production. Simultaneous increases of the two molecules are considered to indicate activated cells arrested at the G_1 stage of proliferation (Brunner *et al.*, 1999). The egg$^+$IgE^{hi} group had significantly higher levels of background Ki67 expression; however CTLA-4 was also significantly raised, thus reflecting immune activation induced Ki67 production. This was in keeping with the highly activated profile of this group reported in Chapter 4 of this thesis. When the egg$^+$IgE^{hi} group was compared with the HIV singly infected egg$^-$IgE^{lo}, some differences were noted in this dually infected subgroup.This group was shown to have high background expression of Ki67 and CTLA4. Cytokine profiling showed that at baseline as well as in response to HIV-1 p24 challenge this group persistently had low and/or undetectable Th_1 cytokines (IL-2, IFNγ and IP-10).

In stimulated assays, all the helminth infected subgroups (the egg$^+$IgE^{hi}, the egg$^+$IgE^{lo} and the egg$^-$IgE^{hi} groups) had potent Ki67 responses in response to challenge with PHA mitogen. However the egg$^+$IgE^{hi} group displayed a tendency towards lower proliferation in response to the HIV-1 p24, streptokinase and *Ascaris* specific antigens. This apparent reduced capacity to proliferate in response to specific antigens (despite retained proliferation to mitogen) concurs with the proposed induction of specific anergy by helminth infections (Maizels and Yazdanbakhsh, 2003, Borkow and Bentwich, 2004), thus impairing effective immune responses to HIV as per the present study hypothesis.

Cytokine production assays also demonstrated results that support the hypothetical negative influence of helminthiasis on immune responses to HIV. The antiviral Th_1 cytokine IFNγ was significantly lower in the baseline (unstimulated) cells from individuals in the egg$^+$IgE^{hi} group. Likewise another Th_1 cytokine, IL-2 showed a lower trend in this group. IL-2 induces lymphoproliferation and differentiation into effector cells (Janeway *et al.*, 2001), therefore its reduction is in keeping with the reduction in antigen-specific proliferation discussed in the preceding paragraph. Furthermore, these two cytokines together with IP-10were undetectable or present at very low levels when cells were stimulated with the HIV-1 p24 antigen in this group. Both IFNγ and IL-2 are known to play a critical role in antivirus immunity, including HIV control (Janeway *et al.*, 2001). These results therefore uphold the proposed negative influence of helminths on effective control of HIV infection.

Further support for our hypothesis was the finding that both high IgE responder groups (egg^+IgE^{hi} and the egg^-IgE^{hi}) had significant levels of the Th_2 cytokine IL-4 and low secretion of the Th_1 cytokine IL-2. These results confirm an inverse association between Th_2 responses (high IgE, increased IL-4) and Th_1 responses in relation to helminth infection. They support the argument that helminths induce Th_2 polarisation at the expense of the protective anti-viral Th_1 responses and this Th1 to Th2 shift has been associated with HIV progression (Clerici and Shearer, 1993, 2001).

Constitutive production of the two proinflammatory cytokines IL-6 and IL-8 was high and fell outside the standard curve range of the Luminex assay and the interpretation of these results could therefore be flawed. Levels of tumor necrosis factor alpha (TNFα), another inflammatory cytokine, were higher in both egg positive groups (egg^+IgE^{hi} and the egg^+gE^{lo}) although this did not reach statistical significance. Induction of NF-κβ by TNF is implicated with a pro-replicative role for HIV (Lawn *et al.*, 2001).

Finally, the modified Th_2 (the egg^-IgE^{hi}) group had highly significantly increased levels of the Th_1 cytokine IFNγ and highest median IL-2 levels, while IL-4 was significantly lower. In keeping with the modified Th_2 phenotype, the regulatory cytokine IL-10 was higher in this group. Most importantly, the individuals in this group produced significantly higher levels of IFNγ in response to HIV-1 p24 challenge. This further endorses our proposition that these individuals maintain or retain immune competence to control HIV infection despite being coinfected with helminths.

7.5 In summary:

While in most instances, no significant differencesw were noted betweenthe egg^-IgE^{lo} and the egg^+IgE^{hi} subgroups, results from this work suggest that individuals with high Ascaris-specific IgE in serum and with *Ascaris lumbricodes* and or *Trichuris trichura* eggs in stools represent a typical Th_2 (as opposed to a modified Th_2) immune response phenotype. These individuals consistently demonstrated evidence for a potentially deleterious influence of helminths on immune responses against HIV. In all the chapters significant findings that concur with the study hypothesis were found within this group.

Briefly, participants in this group showed a tendency towards altered peripheral blood cell composition (lymphocytopaenia, significant eosinophilia), a highly activated immune profile, altered immune response to HIV (a trend towards low antigen-specific lymphoproliferation, and reduction of protective Th_1 cytokine levels) and higher viral load).

While the typical Th_2 responder (egg^+IgE^{hi}) status displayed the most pronounced and detrimental immunological interaction between helminths and HIV, other responder phenotypes to helminth infestation also displayed this deleterious interaction. The presence of stool eggs irrespective of Ascaris specific IgE levels was associated with significantly increased expression of CCR5 on $CD4^+$ cells (implication for increased HIV transmission); higher background TNFα levels (upregulation of proviral transcription of HIV, (Lawn *et al* , 2001)) and significant expression of CD38 on $CD8^+$ cells (suggestive of HIV progression). On the other hand, the Th_2 features (eosinophilia and high Ascaris-specific IgE, irrespective of presence or absence of stool eggs) were also seen to affect different aspects of immune system dysregulation with regards to HIV pathogenesis. These included the finding of higher frequencies of eosinophilia, higher viral loads, higher percentages of individuals with severe immunodeficiency; a stronger inverse correlation between absolute $CD4^+$ counts and $CD8^+CD38^+$ plus reduced Th_1 and increased type 2 cytokines.

Lastly, a prominent finding was that the modified Th_2 responders (egg^+IgE^{lo}) appear to control HIV better than their typical Th_2 counterparts. This was shown by higher proportions of lymphocyte populations, lower viral load plus lower numbers of individuals with advanced immunodeficiency in terms of $CD4^+$ counts, lower Th_2 and higher type 1 cytokines and it confirmed the existence of major phenotypic differences with profound immunological implications between the two groups. These suggest that some individuals may be susceptible to helminth infections but have a modified Th_2 phenotype and control HIV infection better. The apparent improved control of HIV fits with the increased IL-10 (regulatory cytokine) levels in this group and concurs with the alternate theory that regulatory pathways associated with helminth infections may downregulate the pro-replicative inflammatory processes that promote HIV transcription and replication (Lawn *et* al., 2001; Brown *et al*., 2006).

7.6 Concluding remarks

The present study was designed to test the hypothesis that helminths impair immune responses to HIV. The study had limitations, such as possible selection bias in HIV positive support group attendees, a cross sectional design, and the small sample size. Moreover, the fact that HIV is a progressive infection and that there is a lack of information regarding chronicity/duration of HIV infection will further impact on the findings of a cross sectional design of the study. However the significant findings noted despite these limitations are encouraging, thus strongly suggesting existence of immunological interaction between helminthiasis and HIV.

The results presented in this thesis provide evidence that the presence of intestinal parasite eggs in stools of infested individuals represents only a part of the helminth infection phenotype, which can be further delineated by levels of helminth-specific IgE (and IgG_4) (Maizels and Yazbandashksh, 2003). Grouping according to stool egg positivity alone would misclassify infection and non-infection, obscure the recognition of additional phenotypes with major implications for the interpretation of studies addressing immunological effects of co-infections with helminths.

The author concludes that helminth infections may be associated with deleterious effects on the immune responses to HIV in certain groups of susceptible individuals based on the key findings that:

(i)The $egg^{+}IgE^{hi}$ status clearly confers an immunologically compromised immune response to HIV. This status reflects susceptibility to helminth infection and displays negative influences on the response to HIV infection and progression as measured by viral load and decreased $CD4^{+}$ cell count.
(ii) A Th_2 biased profile linked to helminth infection is also associated with some compromised immune response to HIV.
(iii) The presence of nematode stool eggs irrespective of IgE levels also affects some aspects of HIV pathogenesis or immune responses.
(iv) The individuals with stool parasite eggs but low IgE (or modified Th_2 responders) may maintain some immunological resilience towards HIV infection.

The present work has made a contribution to the body of new knowledge in South Africa. The significance of including the serological marker (IgE) to enhance the diagnosis of intestinal nematodes but also to further define immune phenotypes in the context of helminth infection is considered an important finding. This has implications for the design of future studies aimed at analysing helminth coinfection and more importantly, interpretation of such studies.

Recommendations

It is therefore recommended that properly designed and controlled studies be extended and should include serological tests (parasite specific IgE and IgG4) to differentiate the immune phenotypes of helminth infection.

Based on the finding that helminthiasis is in some individuals associated with more advanced HIV infection it is highly recommended that concerted efforts be made to control helminth infections, particularly in those with high risk for HIV infection and in those with known HIV infection. This will make a contribution towards a holistic approach to prevention of and management of HIV infection. Sustained mass deworming of preschoolers, scholars and adults combined with improved sanitation programmes and education campaigns should be implemented to decrease both worm burden in individuals and the number of infected individuals, which would also reduce the overall transmission of helminths, although the present data does not address the immunological effect of deworming on responses against HIV. Further studies that analyze the effect of deworming on co-infected individuals are needed as these have yielded conflicting results. However, it may be beneficial to implement effective helminth control in children and subsequently at regular intervals in settings with a high prevalence of worm infection. This would prevent co-infection with HIV which has now been shown to adversely affect anti-HIV responses. An integrated health care approach that addresses prevention (and where indicated treatment) for both, and other prevalent health care issues, may have major public health benefits.

The effect of helminth infection on other infections, including tuberculosis, should also be evaluated in future studies.

References

Actor J, Shirai M, Kullberg M, Buller R, Sher A, Berzofsky J. 1993. Helminth infection results in decreased virus-specific CD8+ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. *Proceedings of the National Academy of Science of USA.* **90:**948–952.

Adams VJ, Markus MB, Kwitshana ZL, Dhansay MA, van der Merwe L, Walzl G, Fincham JE. 2006. Recall of intestinal helminthiasis by HIV-infected South Africans and avoidance of possible misinterpretation of egg excretion in helminth/HIV co-infection analyses. *BMC Infectious Diseases***. 6 (88):** 1-8.

Anonymous, 2001.The Khayelitsha Task Team (KTT): building health partnerships that work. ISBN 1-919809-26- 0. Brochure published by the Khayelitsha Task Team. Obtainable from the Medical Research Council, Cape Town, South Africa.

Bhattacharyya T, Santra A, Debendra N. *et al*. 2001. Possible approach for serodiagnosis of Ascariasis by evaluation of Immunoglobulin G4 response using *Ascaris lumbricoides* somatic antigen. *Journal of Clinical Microbiology***. 39 (8):** 2991-2994.

Borkow G, Bentwich Z. 2004. Chronic immune activation associated with chronic helminthic and Human Immunodeficency Virus infections: Role of hyporesponsiveness and anergy. *Clinical Microbiology Reviews***. 17(4):** 1012-1030.

Brown M, Kizza M, Watera C, *et al.* 2004. Helminth infection is not associated with faster progression of HIV disease in coinfected adults in Uganda. *The Journal of Infectious Diseases.* **190:**1869-1879.

Brown M, Mawa PA, Kaleebu P, Elliott M, 2006. Helminths and HIV infection: epidemiological observations on immunological hypotheses. *Parasite Immunology***. 28(11):** 613–623.

Brunner MC, Chambers CA, Chan FK, *et al*. 1999. CTLA-4- mediated inhibition of early events of T cell proliferation. *Journal of Immunology.* **162:**5813-5820**.**

Clerici M, Shearer GM. 1993. A TH1→TH2 switch is a critical step in the etiology of HIV infection. *Immunology Today.* **14:**107 11.

Clerici M, Stefano B, Matthew L, *et al.* 2000. Immune activation in Africa is enviromentally-driven and is associated with upregulation of CCR5. *AIDS 1***4 (14) :**2083-2092.

Clerici M, Shearer GM. 2001. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunology Today.* **12:** 575-581.

Elliot MA, Mawa PA, Joseph S, *et al.*, 2003. Association between helminth infection and CD4+ T cell count, viral load and cytokine responses in HIV-1 infected Ugandan adults.*Transactions of the Royal Society of Tropical Medicine and Hygiene***. 97:**103-108.

Freedman AR, Gibson FM, Fleming SC, *et al.* 1991. Human immunodeficiency virus infection of eosinophils in human bone marrow cultures. *The Journal of Experimental Medicine***. 174:**1661-1664.

Gerrard, J. W. 1985. Genetic factors in the development of asthma. *In* E. B. Weiss, M. S. Segal, and M. Stein, editors. Bronchial Asthma- Mechanisms and Therapeutics, 2nd ed. Little Brown, Boston. 24-29.

Godoy-Ramirez K**,** Franck K, Mahdavifar S, *et al.* 2004. Optimum culture conditions for specific and nonspecific activation of whole blood and PBMC for intracellular cytokine assessment by flow cytometry. *Journal of Immunological Methods***. 292 :**1-15.

Gopinath R, Ostrowski M, Justement SJ *et al.*, 2000. Filarial infections increase susceptibility to Human Immunodeficiency virus infection in peripheral blood mononuclear cells in vitro.2000. *The Journal of Infectious Diseases***. 182:**1804-1808**.**

Grossman Z, Meier-Schellersheim M, Paul WE, Picker LJ. 2006. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys*. Nature Medicine***.12(3):**289-95.

Haus M, Heese H De V, Weinberg EG, Potter PC, Hall MJ, Malherbe D. 1988. The influence of ethnicity, an atopic family history and maternal ascariasis on cord blood serum IgE concentrations. *Journal of Allergy & Clinical Immunology*. **82:**179-189.

Hodge G, Hodge S, Han P. 2000. Increased levels of apopotosis of leucocyte subsets in cultured PBMCs compared to whole bloodas shown by Anexin V binding: relevance to cytokine production. *Cytokines.* **12:** 1763.

Janeway CA, Travers P, Walport M, Shlomchik M. 2001. *In:* Schanck D, Austin P, Lawrence E, *et al*. eds. Antigen presentation to lymphocytes. *Immunobiology . The immune system in health and disease. 5th ed. Chapters 1-14.* Garland Publishing. Taylor and Francis group

Kalinkovich A, Weisman Z, Greenberg Z, *et al.* 1998. Decreased CD4 and increased CD8 counts with T cell activation is associated with chronic helminth infection. *Clin. Exp. Immunol.* **114:** 414-421.

Kalinkovich A, Borkow G, Weisman Z, Tsimanis A, Stein M, Bentwich Z. 2001. Increased CCR5 and CXCR4 expression in Ethiopians living in Israel: environmental and constitutive factors. *Clinical Immunology* . **100:**107–117.

Kallestrup P, Zinyama R, Gomo E, *et al*. 2005. Schistosomiasis and HIV-1 infection in rural Zimbabwe: Effect of treatment of shistosomiasis on CD4 cell count and plasma HIV-1 RNA load. *The Journal of Infectious Diseases*. **192.** 1956-1961.

Kamal SM, El Sayed Khalifa K. 2006. Immune modulation by helminthic infections: worms and viral infections. *Parasite Immunology.***28:** 483-496.

Lawn SD, Butera S, Folks TM. 2001. The contribution of immune activation to the pathogenesis and transmission of Human Immunodeficiency Virus type1 infection. *Clinical Microbiology Reviews.* **14(4):** 753-777.

Lawn SD, Karanja DMS, Mwinzi P, *et al*. 2000. The effect of treatment of schistosomiasis on blood plasma HIV-1 RNA concentration in coinfected individuals.*AIDS.* **14:**2437-2443.

Leng Q, Borkow G,Weisman Z,*et al*., 2001. Immune activation correlates better than HIV plasma viral load with CD4 Tcell decline during HIV infection. *Journal of Acquired Immune Deficiency Syndromes.* **27:**389-397**.**

Lilly-white JE, Bundy DAP, Didier JM *et al*.,1991. Humoral immune responses in human infection with the whipworm *Trichuris trichiura*. *Parasite Immunology.* **13:**491-507**.**

Lucey DR, Dorsky DI, Nicholson-Weller A, Weller PF. 1989. Human eosinophils express CD4 protein and bind human immunodeficiency virus gp120. *The Journal of Experimental Medicine.***169.** 327-332**.**

Maggi E, Mazzetti M, Ravina A, *et al*., 1994. Ability of HIV to promote a TH_1 to TH_0 shift and to replicate preferentially in TH_2 and TH0 cells. *Science.* **265 (5169):**244-248.

Maizels RM, Yazdanbakhsk M. 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms.*Nature Reviews Immunology***. 3:**733-744.

Modjarrad K, Zulu I, Redden DT, *et al*., 2005. Treatment of intestinal helminths does not reduce plasma concentrations of HIV-1 RNA in coinfected Zambian adults. *The Journal of Infectious Diseases.* **192:**1277-1283.

Secor WE, Shah A, Mwinzi PM, Ndenga BA, Watta CO, Karanja DM. 2001. Increased density of human immunodeficiency virus type 1 coreceptors CCR5 and CXCR4 on the surface of CD4(+) T cells and monocytes of patients with Schistosoma mansoni infection*. The Journal of Infectious Diseases.* **184:**488–496.

Shapira-Nahor O, Kalinkovinch A, Weisman Z, *et al* ., 1998. Increased susceptibility to HIV-1 infection of peripheral blood mononuclear cells from chronically immune-activated individuals. *AIDS.* **12:** 1731-1733.

Sousa AE, Carneiro J, Meier-Schellersheim M, *et al.* 2002. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *Journal of Immunology*.**169:**3400-3406.

Wolday D, Mayaan S, Mariam ZG, *et al.* 2002. Treatment of intestinal worms is associated with decreased HIV plasma viral load. *Journal of Acqiured Immune Deficiency Syndromes.* **31:**56-62**.**

Zunino SJ, Singh MK, Bass J, *et al.* 1996. Immunodetection of histone H2B epitopes correlates with early stages of apoptosis in activated human T lymphocytes. *American Journal of Pathology.* **149:**653-663**.**

Appendices

Appendix 1. Box and Whisker plots of lymphocyte subsets for HIV-1 negative subgroups

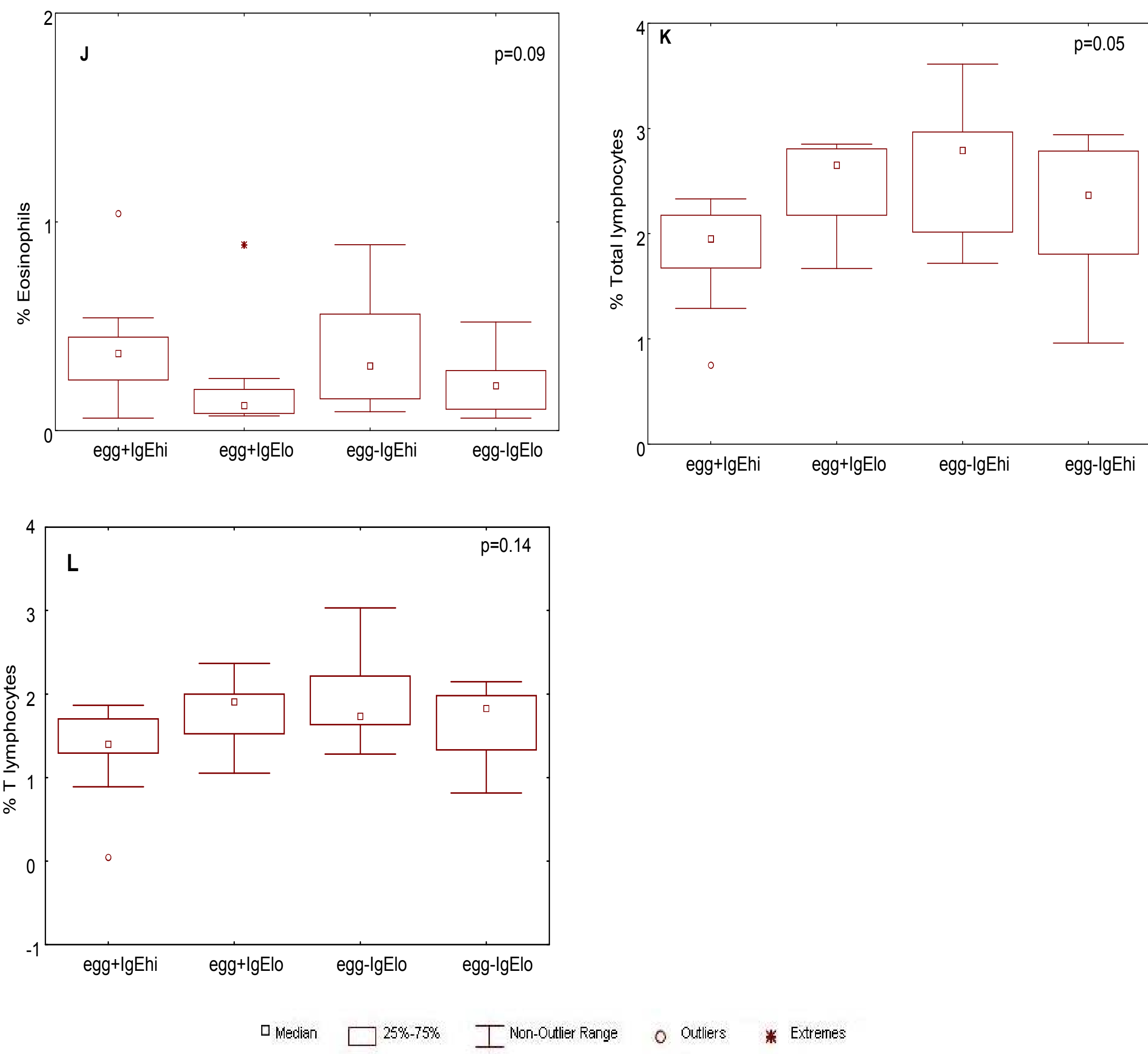


Figure 3. 3 Panels J-L. Kruskal Wallis Box and whisker plots for lymphocyte phenotypes in HIV- groups. Whole EDTA blood was stained with fluorochrome-conjugated monoclonal antibodies to CD45, CD3, CD4, CD8, CD19, CD16 and CD56. Percentages of cells expressing these surface molecules were determined by FacsCalibur flow cytometry using the MultiTest software. Full blood counts were done for eosinophil counts and dual platform calculation of absolute lymphocyte counts. $Egg^{+}IgE^{hi}$ group (n =9) stool helminth egg excretion and elevated *Ascaris lumbricoides* –specific IgE; $Egg^{+}IgE^{lo}$ group (n=9) helminth egg excretion and low Ascaris *lumbricoides* IgE; $Egg^{-}IgEh^{hi}$ group (n=11) No helminth egg excretion and high *Ascaris lumbricoides* IgE; $Egg^{-}IgE^{lo}$ group (n=10) No helminth egg excretion and low *Ascaris lumbricoides* IgE; NK=NK lymphocytes

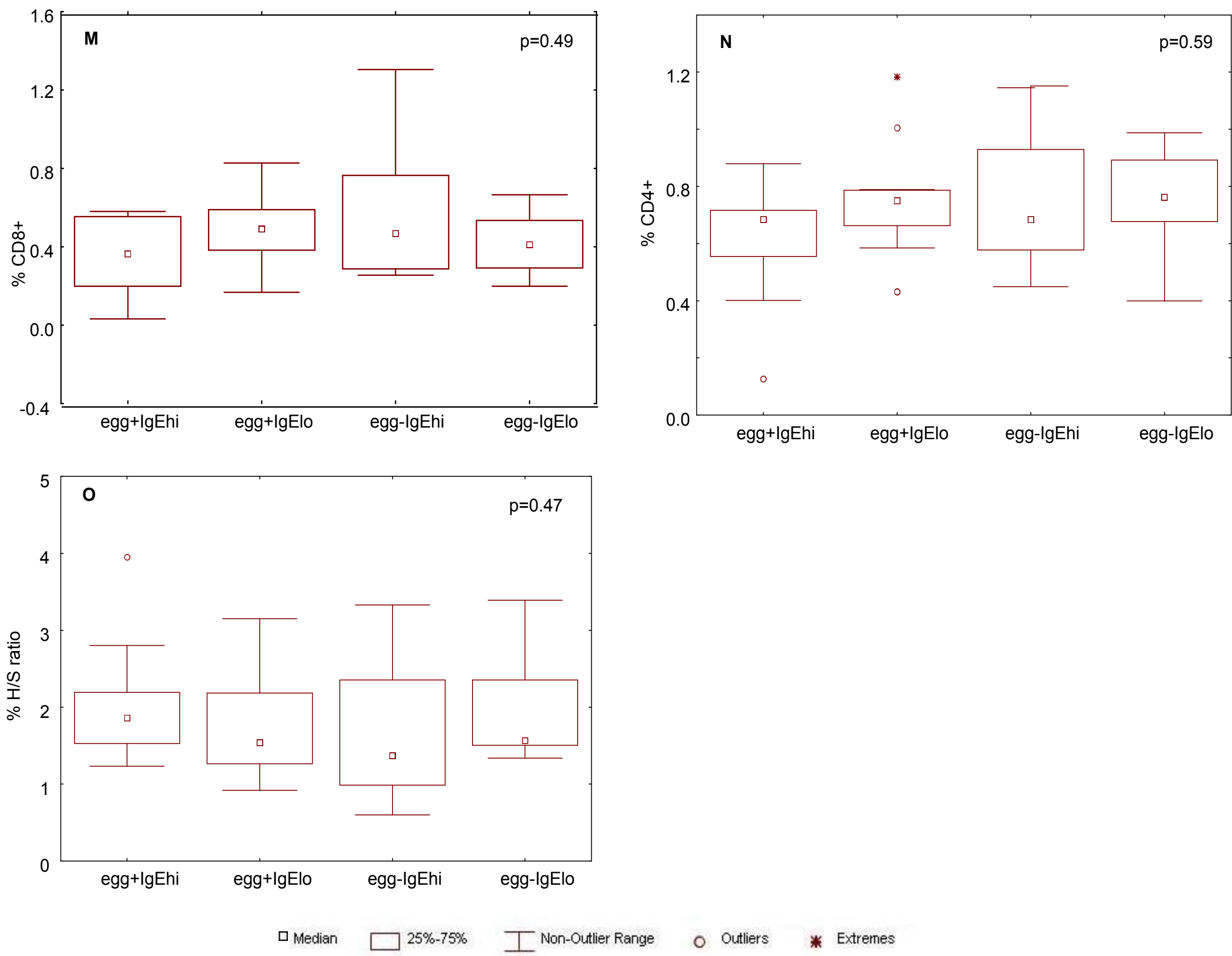


Figure 3. 3 Kruskal Wallis Box and whisker plots for lymphocyte phenotypes in HIV- groups. Percentages of cells that were CD4 positive and CD8 positive were determined by flow cytometry on labeled whole blood. Absolute counts were calculated from these percentages and the full blood counts. Kruskal Wallis ANOVA was used to determine differences between subgroups. H/S Ratio =T helper/T suppressor ratio.

H/S Ratio =T helper/T suppressor ratio.

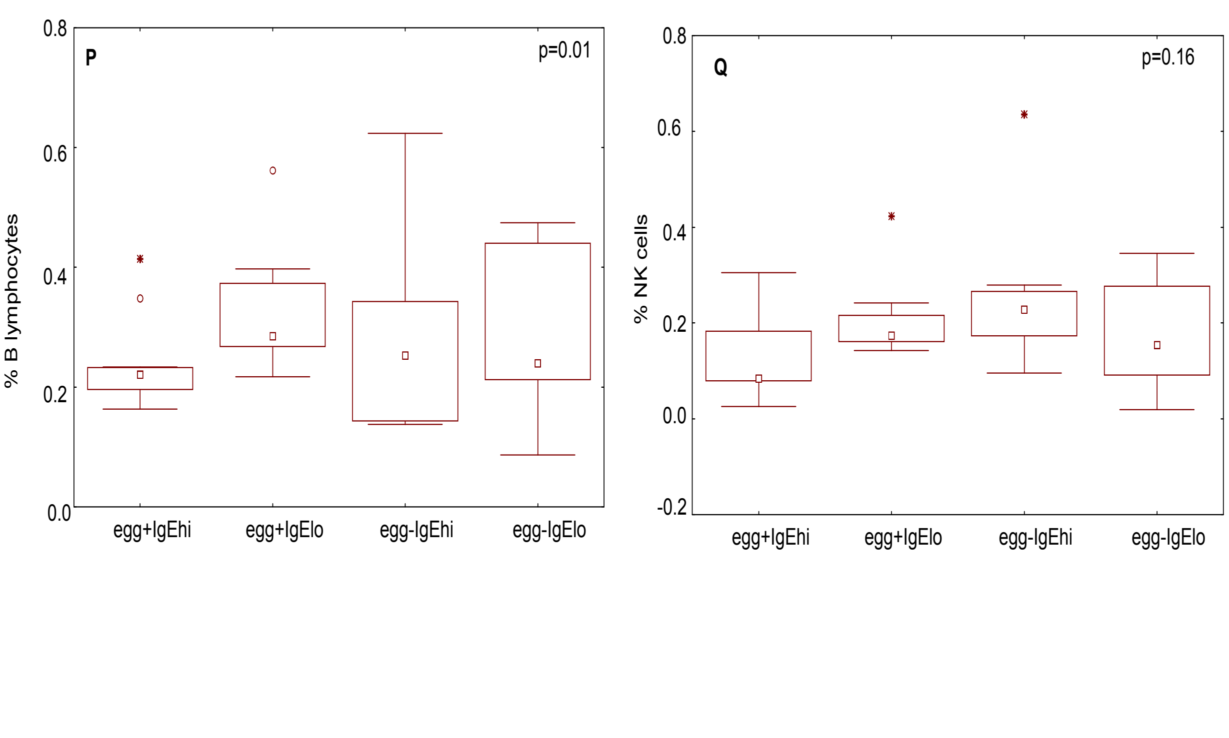


Figure 3. 3 Kruskal Wallis Box and whisker plots for lymphocyte phenotypes in HIV- groups. Percentages of NK and B lymphocytes were determined by flow cytometry on labeled whole blood. Absolute counts were calculated from these percentages and the full blood counts. Kruskal Wallis ANOVA was used to determine differences between subgroups. H/S Ratio =T helper/T suppressor ratio.

Appendix 2. Expression of CCR5 on CD4$^+$ and CD$^+$ cells in HIV$^-$ sugroups

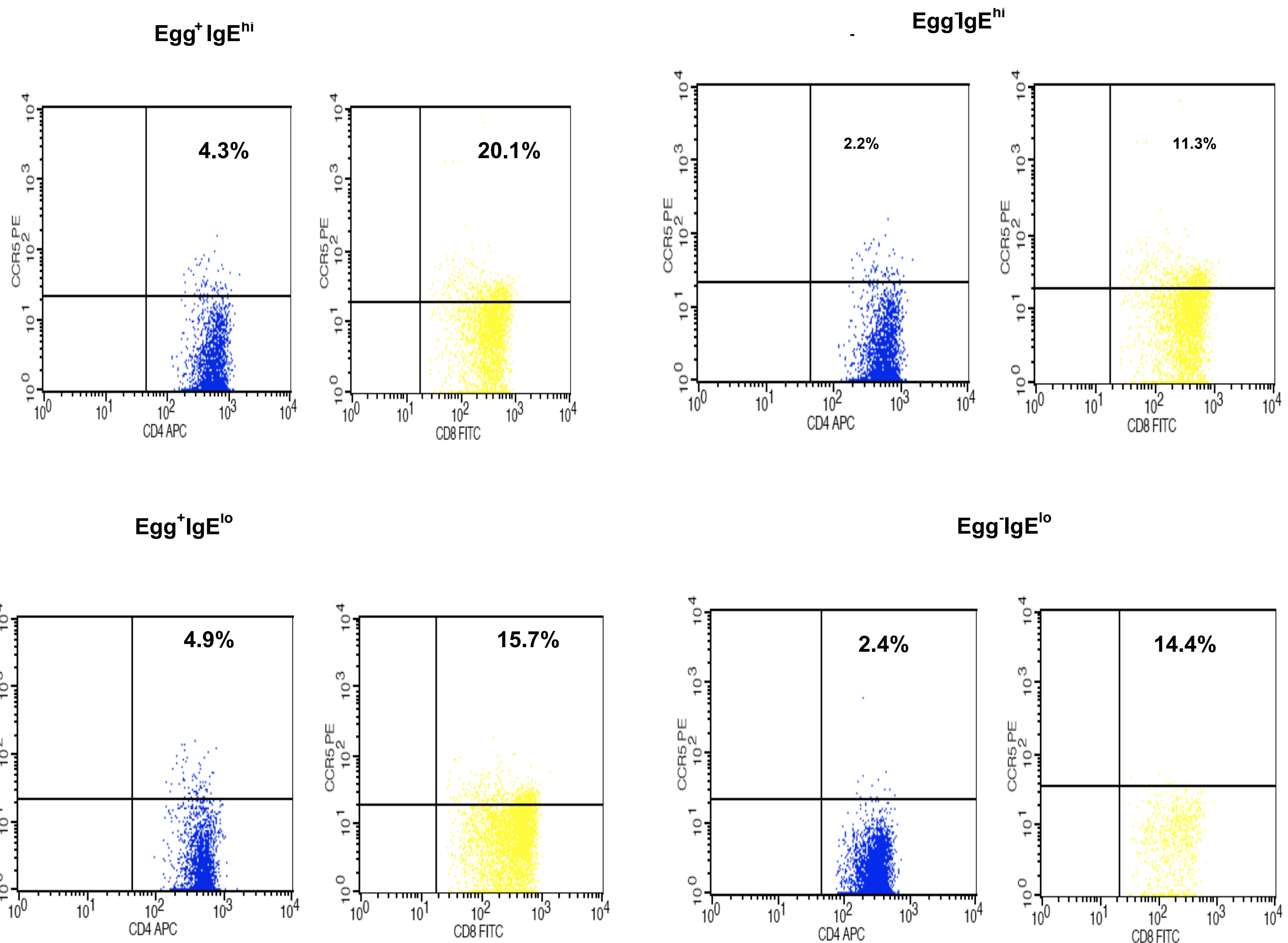


Fig. 4.5 B Expression of CCR5 by CD4+ and CD8+ cells in subgroups of HIV - subjects. Percentage expression of CCR5 on lymphocyte subsets was determined by flow cytometry. Positive cells appear on the right upper quadrants. The percentages represent the median for each group. Each plot is for a representative subject from each group.

Appendix 3 . Expression of CD38 on CD8+ cells in HIV negative subgroups.

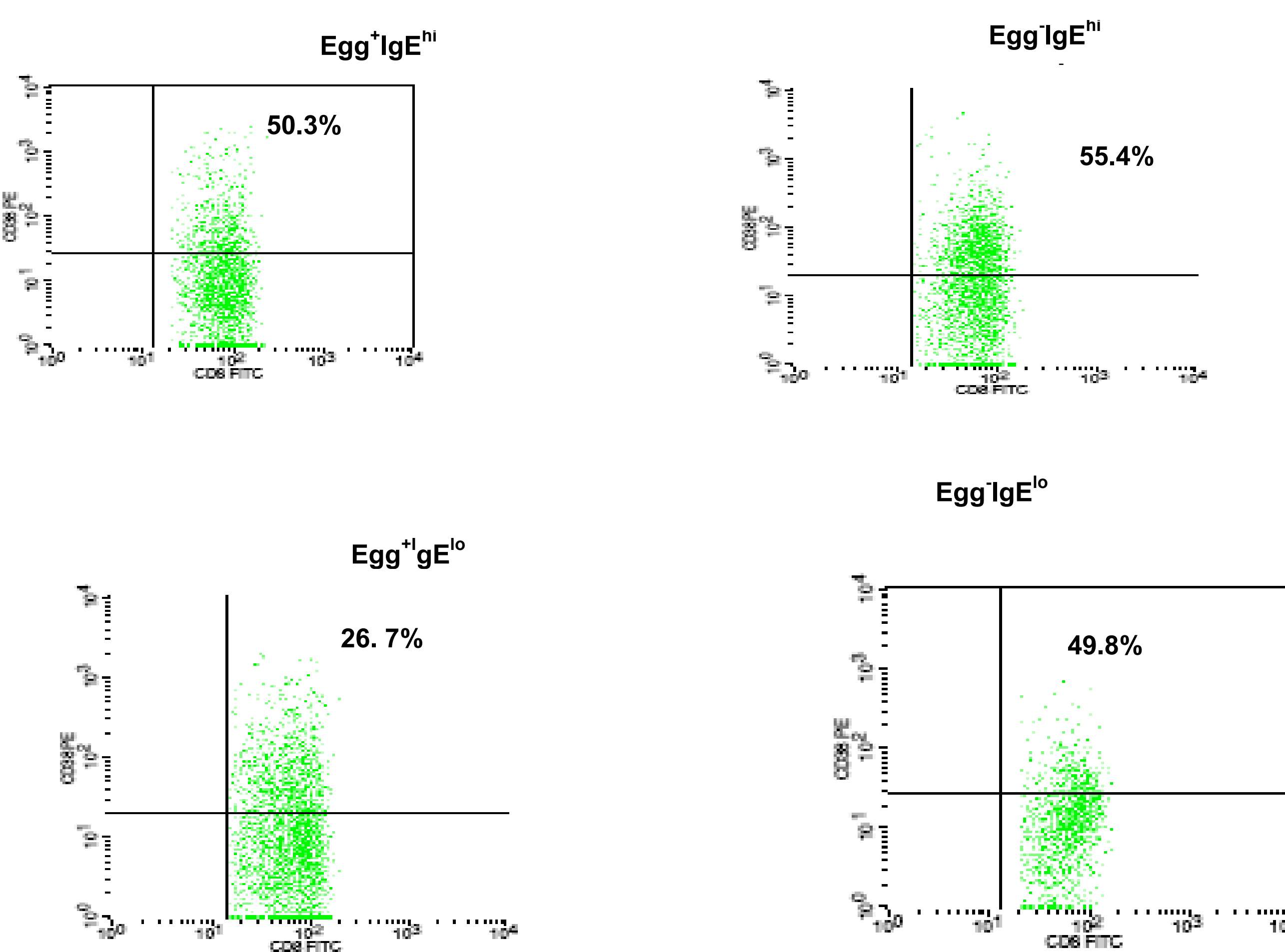


Figure 4.6B Representative plots: Expression of CD38 on CD8^{+} cells in HIV^{-} subgroups. Percentages of CD8 positive cells expressing CD38 were determined by flow cytometry Positive cells appear on the right upper quadrants. The percentages represent the median for each group. Each dot plot is a typical plot of an individual in the respective group.

Appendix 4. Box and Whisker plots for activation markers in HIV$^-$ subgroups.

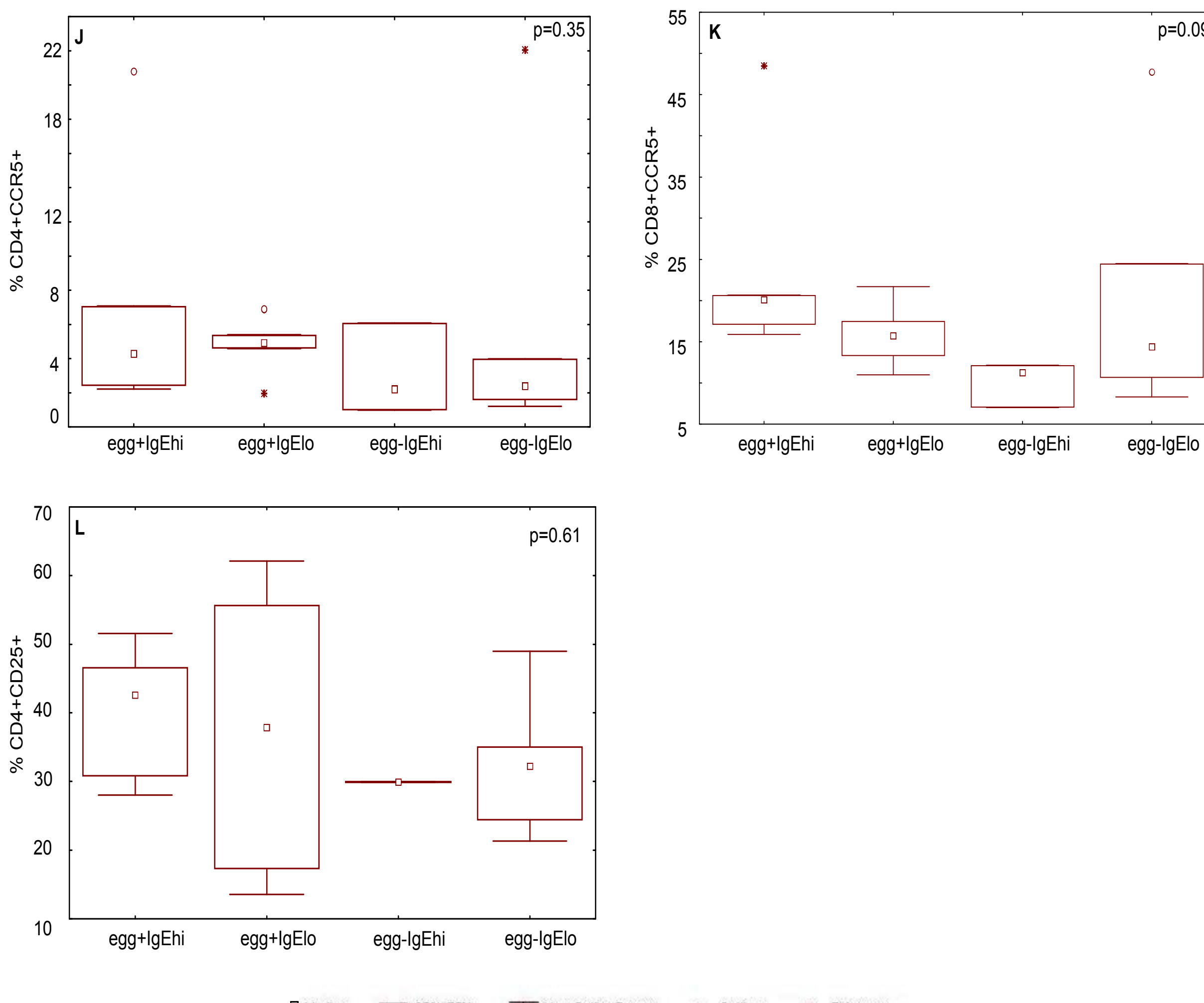


Figure 4.8 Panels J-L Kruskal Wallis Box and whisker plots for lymphocyte surface expression of activation markers on HIV$^-$ subgroups. Ex vivo- cryopreserved, peripheral blood monocytes (PBMC's) were stained with fluorochrome-conjugated monoclonal antibodies to activation marker molecules and the proportions of T cells expressing the particular markers quantified by flow cytometryusing Cellquest^{TM}software. Median percentage expression of these markers were determined for each subgroup. In all panels (J-R), the groups and the sample sizes were: Egg$^+$IgE^{hi} group (n = 5) helminth egg excretion and elevated *Ascaris lumbricoides* IgE; Egg$^+$IgE^{lo} group (n=7) helminth egg excretion and low *Ascaris lumbricoides* IgE; Egg$^-$IgE^{hi} group (n=3) No helminth egg excretion and high *Ascaris lumbricoides* IgE; Egg$^-$IgE^{lo} group (n=7) No helminth egg excretion and low *Ascaris lumbricoides* IgE.

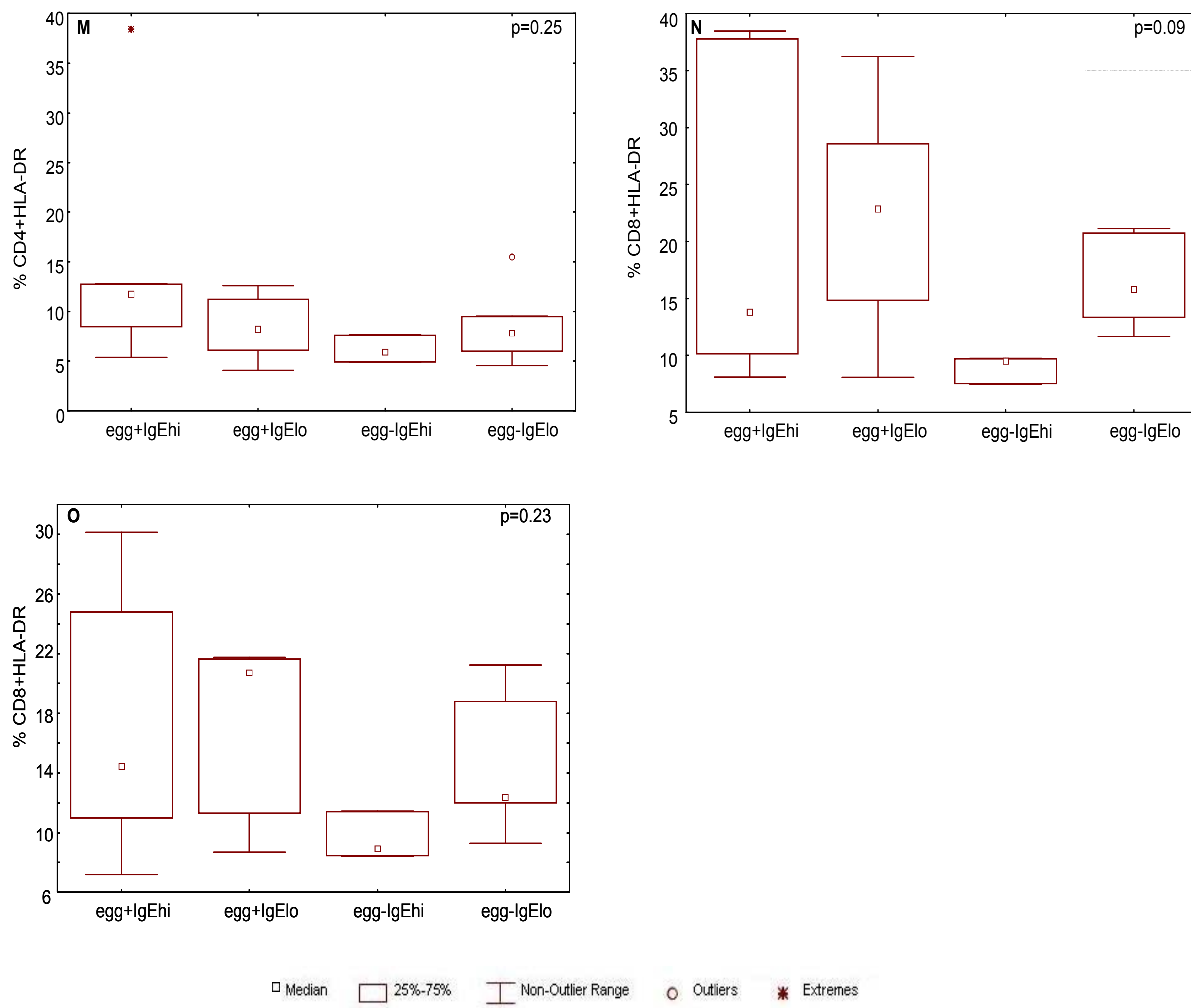


Figure 4.8 Kruskal Wallis Box and whisker plots for lymphocyte surface expression of activation markers on HIV⁻ subgroups.
Expression of surface activation markers (HLA-DR in these panels) was determined on PBMCs of HIV uninfected subgroups by flow cytometry. Significant differences between medians of subgroups were sought by Kruskal Wallis ANOVA.

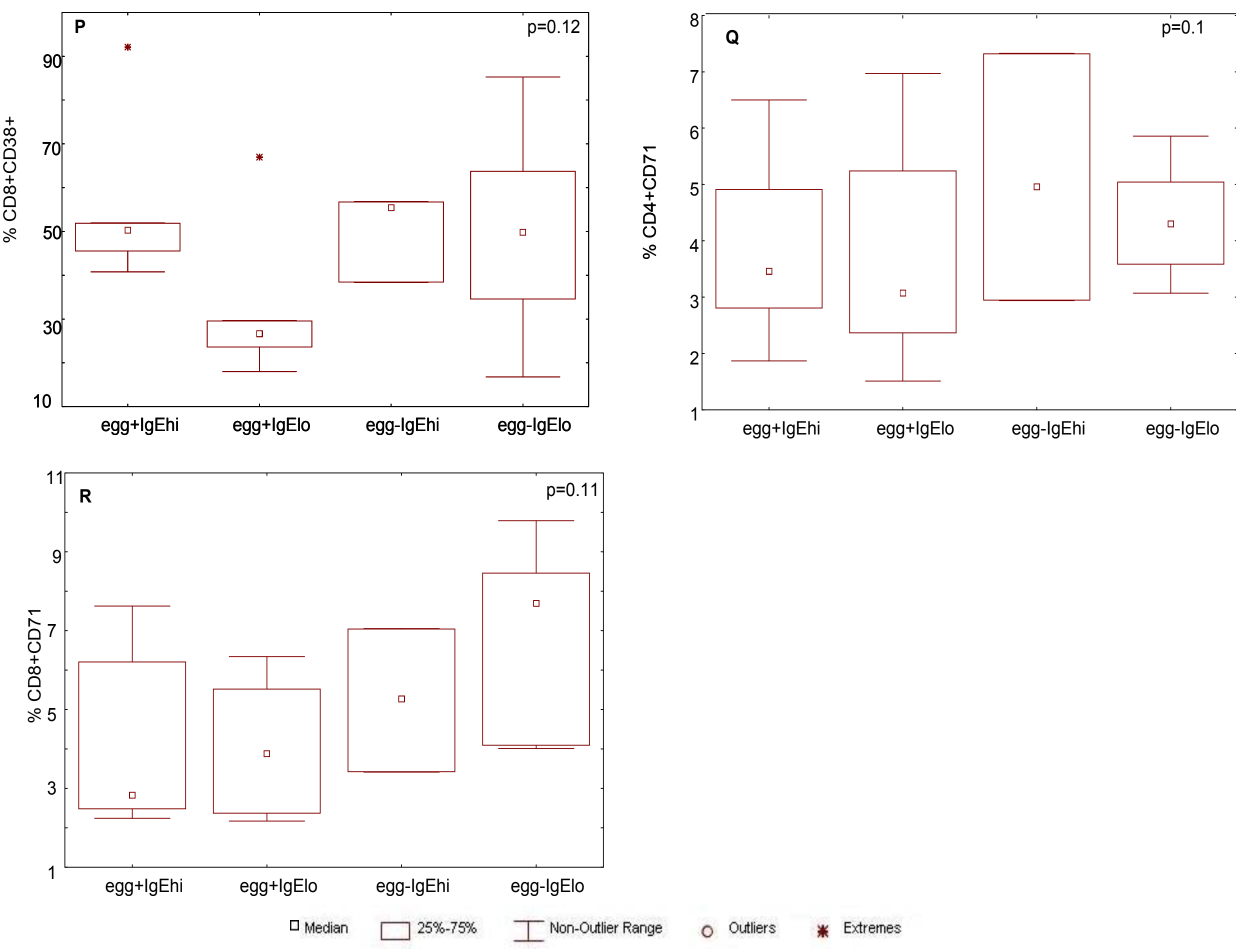


Figure 4. 8 Panels P-R. Kruskal Wallis Box and whisker plots for lymphocyte surface expression of activation markers on HIV^{-} subgroups
Expression of surface activation markers (CD38 and CD71) was determined on CD4 and CD8 lymphocytes of HIV uninfected subgroups by flow cytometry. Significant differences between medians of subgroups were sought by Kruskal Wallis ANOVA.

Appendix 5.
Table 4.5 b. Correlations between activation markers, CD4+ counts and Viral load.

Factor	Group	$CD4^+HLADR^+$ Spearman r (p)	$CD8^+HLA\text{-}DR^+$ Spearman r (p)	$CD3^+HLA\text{-}DR^+$ Spearman r (p)	$CD8^+CD38^+$ Spearman r (p)	$CD4^+CCR5^+$ Spearman r (p)	$CD8^+CCR5^+$ Spearman r (p)	$CD4^+CD71^+$ Spearman r (p)	$CD8^+CD71^+$ Spearman r (p)
$CD4^+$	All (n=40)	-0.4 (0.01)	-0.39 (0.01)	-0.43 (0.01)	-0.44 (0.01)	-0.31(0.06)	-0.03(0.85)	-0.47(<0.01)	-0.37(0.02)
	(Egg^+ IgE^{hi}) (n=10)	-0.56(0.04)	-0.53(0.04)	-0.7 (0.03)	-0.69(0.03)	-0.75 (0.01)	0.01(0.96)	-0.30(0.42)	0.30(0.41)
	(Egg^+ IgE^{lo}) (n=10)	-0.25(0.49)	-0.50(0.13)	-0.54 (0.11)	-0.43(0.21)	-0.72(0.03)	0.47(0.21)	-0.55(0.1)	-0.59(0.07)
	(Egg^- IgE^{hi}) (n=10)	-0.64(0.05)	-0.50(0.1)	-0.59(0.07)	-0.72(0.02)	-0.04(0.91)	-0.34 (0.09)	-0.81(0.02)	-0.83(0.01)
	(Egg^- IgE^{lo}) (n=10)	-0.38(0.27)	-0.35(0.21)	-0.56(0.09)	-0.17 (0.63)	-0.48(0.51)	0.09(0.8)	0.02(0.97)	0.2(0.61)
Viral Load	All**(n=40)**	0.54 (p=0.01)	0.40 (0.48)	0.49 (<0.01)	0.68(<0.01)	0.41(0.01)	0.19(0.25)	0.31(0.07)	0.21(0.19)
	(*Egg^+ IgE^{hi}) (n=10)	0.73 (0.01)	0.31(0.38)	0.49(0.14)	0.07(0.85)	0.5(0.14)	-0.38(0.27)	0.58(0.08)	-0.70(0.03)
	(Egg^+ IgE^{lo}) (n=10)	0.13 (0.7)	0.04(0.9)	0.07(0.86)	0.69(0.03)	0.22 (0.58)	-0.33(0.38)	-0.07(0.85)	0.08(0.82)
	(Egg^- IgE^{hi}) (n=10)	0.54(0.1)	0.35(0.33)	0.65 (0.04)	0.72(0.02)	0.47(0.17)	-0.33(0.34)	0.86(0.01)	0.57(0.11)
	(Egg^- IgE^{lo})	0.30(0.41)	0.23(0.43)	-0.01(0.9)	0.29 (0.41)	-0.32(0.37)	0.14(0.7)	-0.22(0.58)	-0.07(0.86)

Appendix 6 Box and Whisker Plots for proliferation in HIV- subgroups.

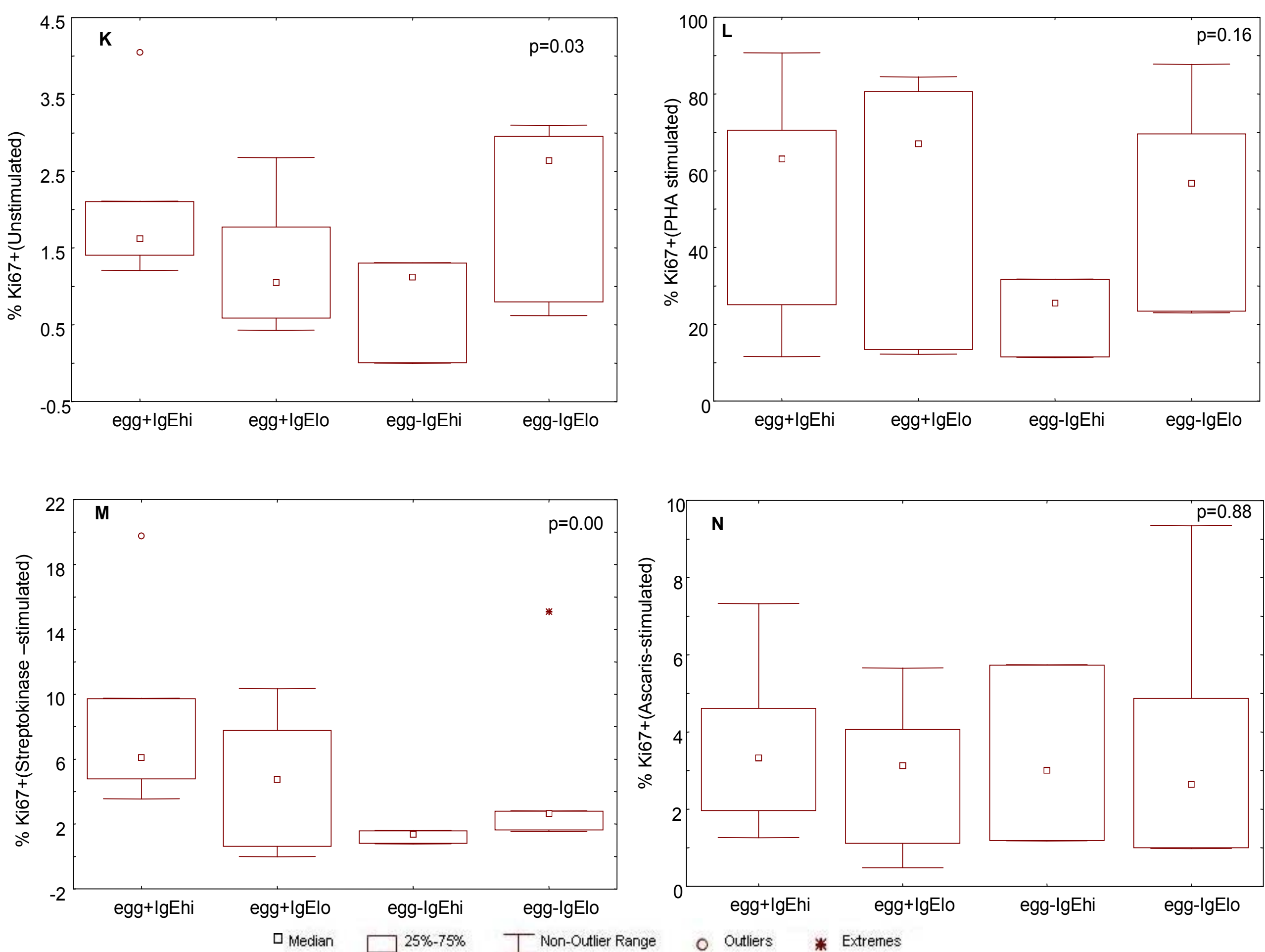


Figure 5 .5 Kruskal Wallis Box and Whisker plots for proliferation antigen - Ki67 and CTLA-4 antigen expression on lymphocytes of HIV- subgroups. Previously-cryopreserved PBMCs were thawed, stained with fluorochrome-conjugated monoclonal antibodies to Ki67, and CTLA-4 and percentages of cells expressing these molecules determined by FacsCalibur flow cytometry using the Cellquet software. In all panels the groups and their n sizes were: Egg^{+}IgE^{hi} group (n = 5) helminth egg excretion and elevated *Ascaris lumbricoides* IgE; Egg^{+}IgE^{lo} (n=7) helminth egg excretion and low Ascaris *lumbricoides* IgE; Egg^{-} IgE^{hi} group (n=3) No helminth egg excretion and high *Ascaris lumbricoides* IgE; Egg^{-}Ig^{lo} (n=7) No helminth egg excretion and low *Ascaris lumbricoides* IgE.
PHA= Phytohaemagluttinin-stimulated cells.

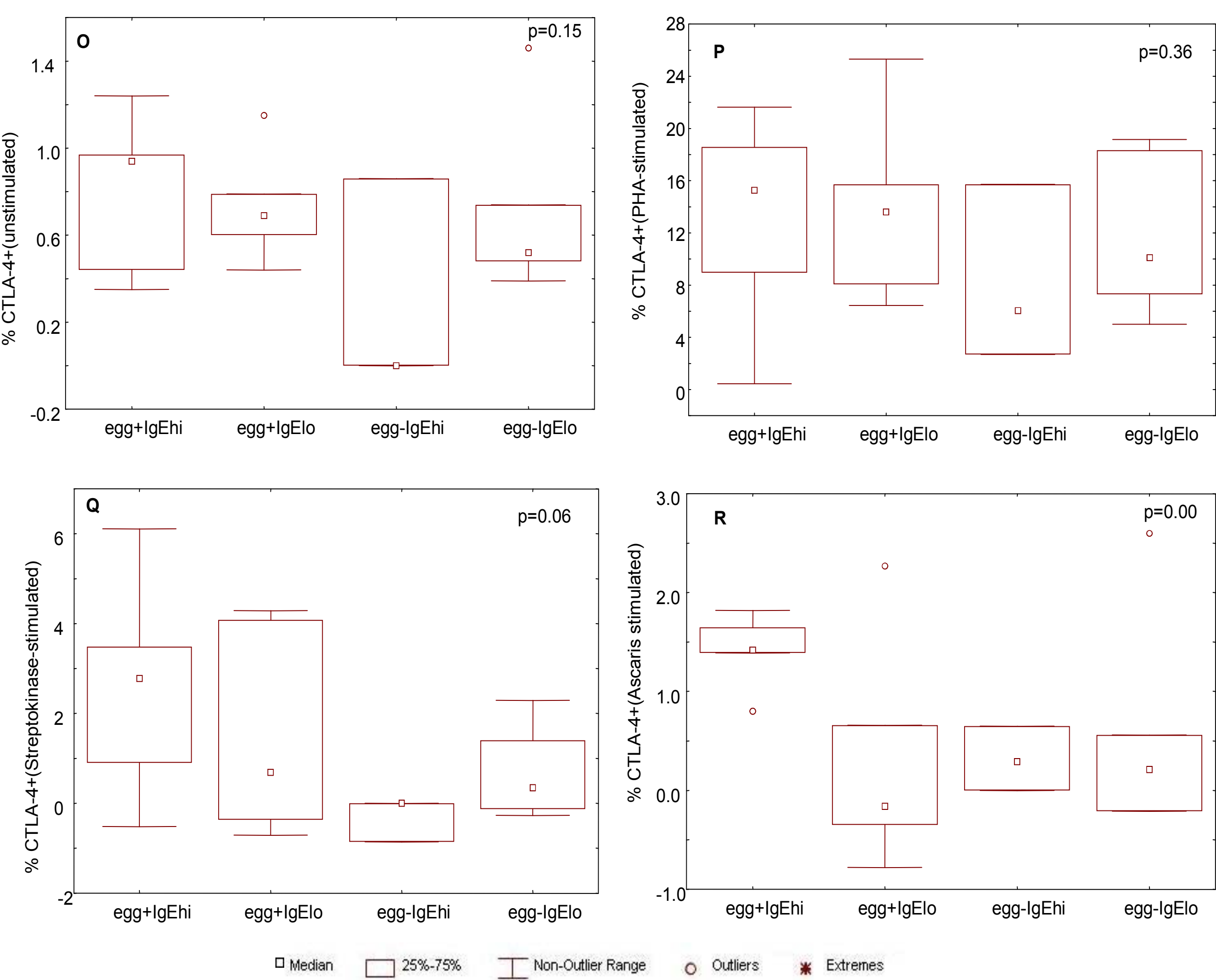


Figure 5 .5 Kruskal Wallis Box and Whisker plots for proliferation antigen - Ki67 and CTLA-4 antigen expression on lymphocytes of HIV- subgroups.
The proliferation antigen Ki67 and regulatory antigen CTLA-4 were assayed by flow cytometry on PBMCs and their median percent expression compared between subgroups of HIV uninfected individuals by Kruskal Wallis ANOVA.

Appendix 7 Preparation of stimulating antigens and media

5.1.1. Mitogens

- Phytohaemagglutinin (PHA). Lectin from *Phaseolus vulgaris)*. (Sigma) Cell culture tested. A 5mg/ml vial was reconstituted aseptically with 5 ml sterile, low endotoxin 1x PBS without calcium or magnesium (BioWhittaker™) made in pour water (Sabax™) to make 1mg/ml stocks. Twenty microliter aliquots were made in sterile vials and kept at -20ºC. Phorbol myristate acetate (PMA) (Sigma). The 1 mg vial was dissolved in 0, 1 ml DMSO and made up to 1ml with sterile, tissue culture grade water to make 1mg/ml. Ten microliter aliquots were frozen at -20ºC and thawed once for use. Final working concentrations of 50ng/ml and 20 ng/ml were made for optimization experiments.
- Calcium Ionomycin (IO) (Sigma). Dissolved in 1ml ethanol to make 1mg/ml and 10 µl aliquots stored at -20ºC. A working concentration of 1000 ng/ml was used. Two combinations of concentrations were used to find the optimal stimulatory one (i) 50 ng /ml PMA and 1ug/ml calcium ionomycin (ii) 20 ng/ml PMA and 1ug/ml calcium ionomycin.

5. 1. 2 Recall antigens

- Streptase (Streptokinase) Aventis. Each vial contained 750 000 I.U. of streptokinase. Since 1 I.U. =0.011ug thus 10ug = 1000 I.U. A 1000 I.U. /ml (10ug/ml) working concentration was made. The stocks were made to be 100x concentrated (100 000 I.U. /ml). The 5ml vial was reconstituted in 7,5ml tissue culture grade PBS to make a 750 000 I.U. /7,5 ml which was 100 000 I.U./ml. Aliquots of 50 µl each were made and stored at -80ºC and thawed once during use. Two concentrations (2 ug /ml or 250 I.U. /ml and 10ug/ml or 1000 I.U/ml) were used to optimize the culture conditions. For each, 2,5 µl and 10 µl were used for each well containing 1 ml volume of culture.
- Purified Protein Derivative (PPD) obtained from Weybridge Veterinary Institute (UK). Reconstituted to 2mg/ml stock in PBS (endotoxin <0.12EU/ml) and aliquots of 400 ug/ml were kept at -20ºC and thawed once for use. A working

concentration of 5 ug /ml was used to stimulate the cultures, for which 12, 5 μl of the 400 ug/ml stock was added to each 1 ml culture. For the 1 ug /ml, 2,5 μl was used.

5.1.3 Medium and Supplements

RPMI 1640 culture medium with glutamax (Gibco,). The complete RPMI 1640 medium with glutamax (cRPMI) contained 10%human male AB serum, the antibiotics and supplements at the concentrations described below.

- No Azide Low Endotoxin monoclonal anti-CD28 (BD Pharmingen). The 0,5mg vial was dissolved in 0,5ml tissue culture grade PBS to make 1mg/ml. To supplement cultures, with 1ug/ml, 1 μl was added to each 1ml culture volume.

- No Azide Low Endotoxin monoclonal αCD49d (BD Pharmingen). Also the 0,5mg vial was dissolved in 0,5ml tissue culture grade PBS to make 1mg/ml and 1 μl added to each 1ml culture volume.
- 2- Mercaptoethanol. (βME) Riedel de Haen. A 1000x stock solution was prepared by adding 2μl of βME into sterile 2,86ml sterile, tissue culture grade (low endotoxin) water. Fifty microliter aliquots were stored at -20ºC for up to a month. These were thawed once. A final working concentration of 50μM (3,5μl/L) was used.
- Penicillin 100U/ml Gibco. A 100x stock was made, aliquoted and stored at -20ºC and each aliquot was thawed once.
- Streptomycin100ug/ml. Gibco. A 1000x stock was prepared and aliquots frozen at -20ºC and thawed once during use.
- Human (male) AB serum. Heat inactivated. US origin. Gibco. The serum was centrifuged at 4ºC at 1500 rpm and the lipoproteins removed. Smaller volume aliquots (5ml) were filtered through 0,22μ acetate filters and were kept at -20ºC until use.

5.1.4 Antigens

- **HIV-1 p24 Antigen** High Purity grade. Research Diagnostics Inc. A recombinant p24 (H x B2 strain produced in *pichia pastoris*) that reacts with AIDS sera and monoclonal antibodies specific for conformational and linear epitopes. The 1mg vial

was reconstituted in 0,36ml of sterile tissue culture grade 0.02M sodium phosphate to make a 2, 8 mg/ml solution. Aliquots of 400ug/ml were made and stored at -80ºC and thawed once during use. Two concentrations (1 and 5 ug/ml) were titrated to obtain the optimum quantity for cell stimulation: 2,5, µl and 12, 5 µl were added to each 1 ml of culture, respectively.

- **PBS-soluble adult *Ascaris lumbricoides* helminth antigens**

Sources:

Two sources of whole *Ascaris lumbricoides* worms were used. The first lot was kindly donated by Natalie Nieuwenhuizen from Professor Frank Brombacher's Immunology Laboratories at the University of Cape Town, South Africa. These had been collected from South Africa. The worms had been washed, sexed and already extracted when they were donated.

The second lot was a kind donation from Professor Jan Bradley of the University of Nottingham, UK. These worms had been collected in Cameroon, West Africa. They had been washed and sexed in the UK and female worms were donated for this study. These were transported on dry ice and immediately stored at -80ºC immediately upon arrival in South Africa.

Preparation of *Ascaris lumbricoides* helminth extracts.

The worms were cut into small pieces in a sterile Petri dish and transferred to 50ml Falcon tube. Approximately 10 ml of sterile, tissue culture grade PBS was added. The slurry was homogenized at 24 000r/minute in a Type 2 homogeniser (KA, Labortechnik, Stakefen, Janke & Kunkel, Gmb).The homogenate was aliquoted into 2ml sterile Eppendorf tubes and centrifuged at 14 000 rpms for 1 hour at 4ºC (until the supernatant was clear).The supernatants were then pooled and filtered through 8um, then through a 0.45um and finally through 0.22um acetate filters. Sterile aliquots were then made and stored at -80ºC.These and endotoxin levels detected. The aliquots were thawed once during experiments.

5.1.5 Determination of protein contents of the Ascaris lumbricoides helminth extract: BCA colorimetric assay

The protein content in the helminth extract was determined using the BCA kit (Pierce) as per the manufacture's instructions. Essentially, a 1: 50 BCA working solution was made by adding 60µl of solution B into 3ml solution A. Protein standards were prepared by making doubling serial dilutions of bovine serum albumin (BSA) in1x PBS to give the following concentrations of protein: 5; 2, 5; 1, 25; 0,625; 0.31; 0,15; 0,08 and 0,04 mg protein. Doubling dilutions of the helminth extract were made in PBS as 1:5; 1:10; 1:20; 1:40 and 1:80. An undiluted sample was also analysed. Two hundred microliters of the BCA working solution were pipetted into 5 ml polystyrene tubes labeled with each standard and sample dilution. Ten microliters of the diluted standards and helminth extract samples (including an undiluted sample) were added to the solution in each corresponding tube and mixed. The tubes were incubated at 37ºC for 30 minutes, and then allowed to cool to room temperature. Absorbance was then read at 540λ. A standard curve was drawn and the corresponding protein concentrations extrapolated.

The endotoxin levels were kindly detected by Ms A. Lynch, University of Cape Town, SA.

5.1.6 Evaluation of antigenecity of the helminth extract : Agarose Gel Diffusion Test

To confirm antigenecity of the extracts, various concentrations (1, 2, 5 and 10ug/ml) of the crude antigens were tested on sera obtained from two participants, HIV-1 negative and positive, who had elevated levels of serum *Ascaris lumbricoides*-specific IgE and excreted *Ascaris lumbricoides* eggs in their stools.

Agarose A Gel Preparation

EDTA (0, 03 g) and 0,75g of Agarose (Seravac, Pharmacia) were dissolved in 100ml of boiling PBS containing 0,1% Sodium Azide. This was aliquoted in 2ml vials, cooled and stored at 4ºC (at which the gel solidified). For the tests, an aliquot of the gel was removed and heated until melted. One milliliter was pipetted onto a glass plate and allowed to cool. Just before the gel set, a Perspex template (with punched holes) was gently lowered onto the agarose gel, sandwiching it between the glass and Perspex surfaces. The prepared plate was left for 4 hours in a humid chamber. For each plate, a central hole, equidistant to 6 other wells was allocated for antigen addition. Ten microliters of the different antigens were

added to the central well, and ten microliters of sera added to the respective surrounding wells. The plate was incubated at room temperature in a humid chamber and allowed to diffuse overnight. Precipitin bands, formed by antigen-antibody binding, were checked for the following day.

5.2 Optimisation of Culture Experiments.

Suitable culture conditions were evaluated for optimal cell activation and cytokine production. This was done by varying the culture period from 6, 12, 24, 48, 72 and 120 hours. The two mitogens were assessed at two concentrations (PHA: 1 and 5 ug/ml) and (PMA 50ng/ml /IO 1ug/ml and PMA 20 ng/ml /IO1ug/ml). Similarly the following recall and specific antigen concentrations were evaluated; PPD (1 ug/ml and 5 ug/ml), streptokinase (250 IU/ml and 1000IU/ml) HIV-1 p24 antigen (1ug/ml or 5ug/ml) and PBS-soluble Ascaris lumbricoides extract (5ug/ml and 10ug/ml). For these experiments, PBMCs obtained from 6 HIV-1 positive and 1 HIV-1 negative participants and 4 presumably healthy controls (laboratory workers) were used. Respective totals of 30, 4 and 19 culture sets were repeated for each of the categories on different days. The cultures were set up as described in 5.3.1 below under varying conditions

Optimisation Procedure

Six plates were set up, each plate for the specific incubation period. Each sample from each donor was designated to 24 wells of a 48-well polypropylene microtiter tissue culture plates (Corning, Costar) and two wells were set aside for the unstimulated cells. Cell suspensions containing $1x10^6$ cells/ml in complete RPMI 1640 (supplemented with 50µM β Mercaptoethanol, 100/ml penicillin and 100µg/lml streptomycin) were added to the designated wells. One microliter each of the 1mg/ml of α CD28 and αCD49d were added to the culture wells. Each stimulant was designated to 4 wells (with each of the 2 concentrations set in duplicates). The plates were covered, mixed at 1000 rpm for 1 minute in a plate shaker and incubated at 37ºC in a humidified 5% CO_2 incubator for 6, 12, 24, 48 ,72 and 120 hours. At the end of each incubation period, the respective plate was removed from the incubator and supernatants were carefully collected from the wells without disturbing the cells. From each well, 4 appropriately labeled Eppendorf

vials of 200 µl supernatants were collected and immediately transferred to a -80ºC freezer for later analysis.

To compare the different time points and stimulant concentration for optimum stimulation, two cytokines Interleukin4 (IL-4) and interferon γ (IFN-γ) were analysed on the collected supernatants using the Quantikine High Sensitivity human IL-4 and IFN γ Elisa kits from RDI. The resulting cell pellets were used to optimize the staining for lymphocyte proliferation markers.
For the above experiments, various concentrations of the antigens and mitogens were tested for optimal stimulation of cultures at different incubation time points.

5.3 Determination of Cultured PBMC's Cytokine responses to stimulants

5.3.1 Preliminary experiments

While culture conditions were being optimized, supernatants were collected from unstimulated and stimulated cultures and frozen at -80°C. At a later stage, these were retrieved and analysed for cytokine [Interferon gamma (IFNγ) and Interleukin 4 (IL-4)] production. Eighty two samples were analysed for the production of both IFNγ and IL-4 after culture with various mitogens and antigens for different periods of time ranging from 6 hours to 6 days. The Quantikine human Interferon gamma (IFNγ) and Interleukin 4 (IL-4) High Sensitivity (HS) Elisa kits purchased from Research Diagnostics Systems (RandD) were used. Essentially the manufacturer's instructions were followed without modifications.

5.3.2 The Quantikine HS human IL-4 Elisa

The assay employs the quantitative sandwich technique with a monoclonal anti-IL-4 precoated onto the microplate. Any IL-4 present in samples binds to the immobilized antibody. After washing away unbound material, a second enzyme-linked polyconal anti-IL4 binds to the IL-4 and a substrate added after several washes. The amplifier solution is added to develop a colour that is proportional to the amount of bound IL4. Colour intensity is measured.

Brief Procedure

Reagents and standards were prepared as instructed in the manual. Then serial dilutions of the standard were made to produce concentrations of 16pg/ml; 8 pg/ml; 4 pg/ml; 2 pg/ml; 1 pg/ml; 0, 5 pg/ml and 0, 25 pg/ml. Fifty microliters of assay diluentHD1-6 was added to each well of the microplate, mixed well and 200µl of each supernatant and standard added (in duplicates). The plate was then covered with the adhesive strip and incubated for 3 hours at room temperature (RT). The plate was then washed 4 times with the wash buffer using the ComburTip multidispenser and completely removing the wash buffer by carefully rapping the inverted plate and blotting in several layers of clean paper towels. Two hundred µl of antibody-enzyme conjugate were added to each well and the sealed plate incubated for 2 hours at RT. The plate was washed 4 times as above and 50µl of substrate solution added. A further 1 hour incubation at room temperature preceded the addition of the amplifier solution and 30 minute incubation. The absorbance was then read within 30 minutes in the Elisa microplate reader at 490nm and wavelength correction set to 650nm. A standard curve is drawn by the computer using the concentrations vs. absorbance obtained. The concentrations of the samples then exported to the Excel software.

5.3.3 IFNγ Elisa.

A sandwich technique employing a polyclonal anti-IFNγ antibody coated onto the plate and a secondary, polyclonal antibody conjugate binds to the bound IFNγ molecules, which are then quantitated by colorimetry.

Procedure in brief

The instructions for reagents and working standard preparation were adhered to. One hundred microliters of assay diluent RD1-51 were added to each well and 100 µl of samples and standards added. The plate was covered with adhesive strip and incubated for 2 hours at RT in the dark. The well contents were decanted by quickly flicking the plate, blotting it onto absorbent paper and then filling it with 400 µl of wash buffer using the ComburTip multidispenser. The wash procedure was repeated 4 times. The anti-IFNγ enzyme conjugate (200µl) was added, the plate covered with adhesive sealer

incubated for 2 hours at RT in the dark. The unbound conjugate was decanted and the wash procedure repeated 4 times. Two hundred µl of substrate was added and plate incubated for 30minutes at RT protected from light. Fifty µl of stop solution was added to all wells and absorbance read at 450nm within 30minutes, with wavelength correction at 540nm in an Elisa plate reader. A standard curve is drawn by the computer using the concentrations vs. absorbance obtained. The concentrations of the samples then exported to the Excel software.

Optimization of Ki67 staining procedure.

The procedure, adapted from BD Biosciences Pharmingen Technical Documents 70-36624K (http://www.bdbiosciences.com/immunocytometry_systems/) was undertaken with various modifications to optimize the method for use in this study.

First an attempt was made to detect viable, proliferating cells by including Propidium Iodide staining.

A 5.2.1 Assessment of viable, proliferating lymphocytes: Propidium Iodide / anti-Ki67 staining

Samples: Sets of cell cultures of PBMC's isolated from 19 laboratory controls, 30 HIV positives and 4 HIV negatives were established for optimization experiments.

For each participant, cells were either cultured without stimulants or challenged with the various mitogens and antigens to optimize the culture conditions and then to perform the actual experiments. Supernatants (800 µl) were carefully removed from cells that had been cultured with or without antigens for six days (as described in section 5.2.1.2 above).The cells were resuspended in the remaining 200 µl of RPMI medium. Ten microliters of cell suspension was removed from each well and enumerated using the counting chamber. Aliquots containing $1x10^6$ cells/ 100 µl were transferred to 50ml polypropylene tubes. Five milliliters of cold 80% ethanol was added dropwise, while vortexing the cell suspensions. The cells were then fixed and permeabilised by incubation for at least 2 hours at -20ºC (in most cases were incubated overnight- they could be used up to 60 days after fixing). Forty milliliters of wash buffer (1X PBS with 1% fetal calf serum and 0,09% NaN_3, pH 7.2) was added to the fixed cells and the tubes centrifuged at 1000 rpm (with the brake on) for 10 minutes. The supernatant was carefully aspirated and discarded. A further wash-centrifugation step was repeated one more time and all supernatant completely removed. The cells were resuspended in 100 µl of wash buffer and transferred to designated wells containing 20 µl of anti Ki67-FITC conjugate (BD Pharmingen) in 96-well v-bottom microtiter plate. The sample-antibody

solutions were mixed in a plate shaker at 1000 rpm for 30 seconds and incubated on ice for 45 minutes in the dark. The plates were then centrifuged at 1500 rpm for 3 minutes at 4ºC with the brake on and the supernatant carefully aspirated by vacuum. (Meticulous effort was taken not to disturb the pellets as these fixed cells did not sediment firmly). One hundred and eighty microliters of wash buffer was added to each well and the plate centrifuged as above. The cells were resuspended in 150 µl of wash buffer and 10 µl of 1mg/ml Propidium Iodide (Sigma) in water was added. The stained cells were transferred to 12x75mm FACS tubes (BD) and analysed on the FACS Calibur ™ Neon-Argon dual laser flow cytometer. All experiments contained control wells including unstained cells only, unstimulated cells and antiKi67-FITC only; PHA-stimulated cells with anti KI67-FITC, PHA-stimulated cells with Propidium Iodide only as well as cells and the IgG1κ isotype control). Unstained cells were used to set the PMT forward and side scatter detector voltages as well as the threshold.

Gating.

A dual parameter dot plot for side scatter vs forward scatter was set up to distinguish the lymphocyte region1 (R1). A second dot plot featuring Propidium Iodide vs anti Ki67-FITC staining was used to distinguish viable cells expressing the KI67 antigen in region 2 (R2). From the two regions R1 and R2, a histogram plot for all viable lymphocytes expressing Ki67 was drawn.

A 5.2.2 Discrimination of proliferating lymphocytes by CD3 Staining

A second modification of the method aimed at improving the recovery of lymphocytes. It included an anti CD3 surface staining step before the antiKi67 staining.

Modified Procedure

Ten microliter aliquots of the cultured cells were enumerated. Twenty microliters of anti-CD3- PerCP (BD Pharmingen) conjugate were pipetted into designated wells of the 96-well v-bottom plate. The enumerated cells were adjusted to contain $1x10^6$ cells in 100 µl and dispensed into the respective wells containing the antibodies. The plates were incubated on ice for 45 minutes in the dark, after which they were centrifuged and supernatants aspirated. One hundred and eighty microliters of wash buffer was added to resuspend the cells and the plates centrifuged at 1500rpm at 4ºC for 3 minutes. The pelleted cells were resuspended in 100 µl wash buffer, transferred to 50ml polypropylene tubes, fixed, and stained for Ki67 as above.

Flow cytometer setting was done as described earlier

Gating:

A side scatter vs forward scatter dot plot was drawn to distinguish the lymphocyte region R1. A second dot plot featuring CD3 vs anti Ki67-FITC staining was used to distinguish all lymphocytes expressing the Ki67 antigen in region 2 (R2). A third region (R3) differentiating viable lymphocytes positive for Ki67 staining was established on the PI vs Ki67 dot plot. From the three regions R1, R2 and R3, a histogram plot for all viable lymphocytes expressing Ki67 (CD 3vs anti K167) was drawn on the gated lymphocytes. This gating strategy was found unsuitable because of the broad spectral overlap between Propidium Iodide and the flourochromes in FL1, FL2 and FL3 chanels and the CD3 light scatter was poor.

A 5.2.3 Improving the side scatter properties: 67% Ethanol/ paraformaldehyde fixation

The third modification involved substitution of 67% ethanol fixative and a paraformaldehyde incubation step to permeabilise the membrane. The aim was to improve the quality of the CD3 gate as the light scatter properties were altered on the alcohol fixed cells.

Procedure

Cell cultures were enumerated and stained for surface expression of CD3 as described above.. The stained cells were washed in PBS and transferred into 15 ml polypropylene tubes. The cells were fixed by dropwise addition of 0,5ml of 67% ethanol and incubated at 4ºC for 30 minutes. Fixed cells were centrifuged at 1500 rpm at 4ºC with the brake on, all supernatant alcohol discarded and 5ml 1XPBS added. The centrifugation step was repeated and supernatant aspirated and discarded. The pelleted cells were resuspended in 0,5ml of 1% paraformaldehyde in PBS containing 0.01% Tween 20 (preparation method in Appendix 1) and incubated for 30 minutes at room temperature. Then the tubes were centrifuged at 1500 rpm at 4ºC with the brake on, supernatant discarded and washed in PBS with the repeat centrifugation. The cells were resuspended in staining wash buffer and stained for intracellular Ki67 expression as described above, with the exclusion of Propidium Iodide staining. The stained cells were analysed on the FACS Calibur™ flow cytometer. After setting the instrument PMT voltages, the threshold and isotype controls, spectral overlap was compensated for between FL1 and FL2 plus FL2 and FL3.

Gating

The lymphocyte region 1 (R1) on the side scatter vs forward scatter dot plot was created. Region 2 for CD3 positive lymphocytes was set on the side scatter vs CD3 plot. Ki67 expression was sought on the third histogram plotting all cells expressing Ki67 gated on R1 $^{+}$R2.

Staining of CD3 staining could not be improved as the side scatter properties were still poor.

A 5.2.4 Anti-Ki67 and anti- CTLA-4 staining

The final modified staining included staining for the downregulator of T cell stimulation – CTL-A4. Surface staining for CD3 expression was excluded as this staining proved unsuccessful. PBMC's from 40 HIV-1 positive participants had each been cultured unstimulated or in the presence of PHA, HIV-1 p24, Streptokinase and *Ascris lumbricoides* antigens. Control samples from 22 HIV-1 negative participants were cultured unstimulated, or challenged with PHA, streptokinase and *A. lumbricoides* antigens. For each sample, 5 wells of a 96 v-bottom plate were designated for the respective stimulant. Twenty microliters of each monoclonal antibodies to Ki67 (conjugated to FITC) and CTLA-4(APC) were carefully pipetted into the bottom of all designated wells. After removal of supernatants, the cell cultures (from 2.7.3) were enumerated and 100 µl aliquots adjusted to contain $1x10^{6}$ cells. The 100 µl aliquots were pipetted into their corresponding wells containing the antibodies. The solutions were mixed by shaking the plates in the shaker at 1000rpm for 30 seconds. Plates were then incubated on ice for 45 minutes in the dark. The plates were then centrifuged at 1500 with the brake on at 4ºC for 3 minutes and supernatant carefully aspirated. One hundred and eighty microliters of wash buffer was dispensed into each well and plates centrifuged as above.

The pellets were resuspended in 180 µl of wash buffer and transferred to FACS tubes containing 450µl of wash buffer. Flow cytometric analysis of the samples then followed. Control samples wells were included: IgG1 κ FITC and IgG_2a APC isotype controls, unstained cells, stimulated and unstimulated cells stained with anti-Ki67 FITC only, anti-CTLA-4 APC only and with both sets of antibodies. The instrument PMT voltages and threshold were set. The isotype control samples were run and the negative population set using a sample from a HIV-1 negative participant.

Gating.

In the side scatter vs forward scatter dot plot, the lymphocyte region was established. For each participant, the unstimulated tube was used to set the gating region and was left unchanged for all subsequent tubes of stimulated cells. Dotplots for Ki67 vs CTLA-4 were drawn and histogram plots were drawn with markers separating the negative and positive cell populations, each for Ki67 and CTLA-4 fluorescence intensity as in Figure5.1.

Appendix 8 Ethical Clearance Document: SA MRC

Ethics Committee

P.O. Box 19070, Tygerberg 7505, South Africa.
Francie van Zijl Drive, Parow Valley 7500, Cape Town.
Tel: +27 (0)21 938 0341, Fax: +27 (0)21 938 0201
E-mail: adri.labuschagne@mrc.ac.za
http://www.sahealthinfo.org/Modules/ethics/ethics.htm

28 March 2002

Dr JE Fincham
Helminths
MRC Cape Town

Dear Dr Fincham

RE: Monitoring the immune profiles of adults co-infected by HIV and intestinal helminths, during a series of anthelmintic treatments

Thank you for your responses to the Ethics Committee, dated 15 and 18 March 2002. Prof Bhoola and I have approved the modifications. I am pleased to inform you that ethics approval is now granted for the study.

Wishing you well with your research.

Yours sincerely

PROF PE CLEATON-JONES
CHAIRPERSON: MRC ETHICS COMMITTEE

Appendix 89.Ethical Clearance Stellenbosch University

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

14 April 2004

Prof G Walzl
Departement Geneeskundige Fisiologie en Biochemie

Geagte prof Walzl

NAVORSINGSPROJEK: "PROSPECTIVE, PLACEBO-CONTROLLED TRIAL OF DEWORMING OF HIV INFECTED INDIVIDUALS"
PROJEKNOMMER : N04/02/045

U aansoek om registrasie en goedkeuring van bogenoemde projek het op 10 Maart 2004 voor die Komitee vir Mensnavorsing gedien. Die Komitee het versoek dat 'n hersiene en herformuleerde protokol voorgelê moet word vir evaluering.

'n Gewysigde aansoek is voorgelê en die projek is finaal goedgekeur op 7 April 2004. Die projek is nou geregistreer en u kan voortgaan met die werk. U moet asseblief in verdere korrespondensie na bogenoemde projeknommer verwys.

Ek vestig graag u aandag daarop dat pasiënte wat deelneem aan 'n navorsingsprojek in Tygerberg-hospitaal nie gratis behandeling sal ontvang nie aangesien die PAWK nie navorsing finansieël ondersteun nie.

Die verpleegkorps van die Tygerberg-hospitaal kan ook nie omvattende verpleeghulp met navorsingsprojekte lewer nie weens die swaar werkslading waaronder hulle reeds gebuk gaan. Dit kan dus van 'n navorser verwag word om in sulke gevalle privaat verpleegkundiges te verkry.

Met vriendelike groete

CJ VAN TONDER
NAVORSINGSONTWIKKELING EN –STEUN (TYGERBERG)

CJVT/cjvt

Fakulteit Gesondheidswetenskappe • Faculty of Health Sciences

Verbind tot Optimale Gesondheid • Committed to Optimal Health

Afdeling Navorsingsontwikkeling en -steun • Division of Research Development and Support
Posbus/PO Box 19063 • Tygerberg 7505 • Suid-Afrika/South Africa
Tel: +27 21 938 9207 • Faks/Fax: +27 21 933 6330
E-pos/E-mail: cjvt@sun.ac.za

Appendix 10. Correspondence for Ethics Waiver –UKN Ethics

From: Dereshni Ramnarain [Ramnaraind@ukzn.ac.za]
Sent: 19 March 2009 09:39 AM
To: Lungi Kwitshana
Subject: RE: FW: Ethics approval

Dear Lungi,

The Chair of the Biomedical Research Ethics Committee, Prof Wassenaar, grants you a waiver of UKZN ethics approval. This is granted on the basis of recognising the MRC and University of Stellenbosch approvals on a reciprocal basis.

Regards,
Dereshni

****

Dereshni Ramnarain
Senior Administrator: Biomedical Research Ethics
Research Office: Govan Mbeki Building
Westville Campus
University of KwaZulu-Natal
Tel: +27 31 260 4769
Fax: +27 31 260 4609
Email: ramnaraind@ukzn.ac.za