Phenotypical and functional characterisation of HIV-specific T cell responses in HIV negative and HIV infected volunteers immunised with GTU® MultiHIV B DNA vaccine

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Declaration of Originality

This work is my own, and all other work included has been appropriately referenced.

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Phenotypical and functional characterisation of HIV-specific T cell responses in HIV negative and HIV infected volunteers immunised with GTU® MultiHIV B DNA vaccine

Dr Goli Haidari, Imperial College London

Abstract

Over the last 30 years, HIV-1 has transformed from an untreatable fatal infection to a chronic condition susceptible to over thirty antiretroviral agents. Although the overall incidence of HIV has declined during the last decade, HIV prevention and treatment still remain key public health priorities worldwide. A vaccine could address both HIV prevention and be utilised therapeutically.

This MD(Res) thesis hypothesizes in both HIV negative and HIV infected individuals, the quality of HIV-specific T cell immunity induced by the GTU® MultiHIV-B Clade DNA vaccine is influenced by the route of vaccination, and has antiviral function in both cohorts. The phenotypical T cell response is presented using IFN-ɣ ELISpot assays on frozen peripheral blood mononuclear cells (PBMCS) stimulated by peptide pools matched to the vaccine to show magnitude, breadth and duration of response, and the functional response is presented using an *in vitro* viral inhibition assay (VIA) as a marker of CD8+ cytolytic activity.

In the first trial, 30 HIV uninfected volunteers were randomised to receive DNA vaccination administered either intradermally (ID), or transcutaneously (TC) in combination with intramuscular delivery (IM), or with electroporation (EP) following IM vaccination. Results showed in the EP+IM group the magnitude, breadth and duration of response was significantly greater than in other groups. In the VIA, all groups showed viral inhibitory activity to at least one virus, with the greatest number of participants with detectable HIV-specific CD8+ cells inhibiting virus in the EP+IM group.

In the second cohort the trial design was modified based on results from the first trial, and 30 HIV infected volunteers on ART were randomised to receive the same vaccine either TC+IM or EP+IM with a further randomisation to receive the vaccine or placebo. The results from T cell ELISpots showed pre-existing immunity to peptide pools in most participants at baseline, but no significant differences either between or within groups or at the primary end point. The VIA was substantially modified for this HIV-infected cohort and showed high levels of pre-vaccination inhibition suggesting some preservation of immune function in these volunteers, although there was little change in viral inhibition at the primary end point.

To summarise, this data indicates that in an HIV uninfected cohort DNA vaccination induced HIV-specific T cell responses particularly using electroporation to enhance IM injection. However, IFN-ɣ alone could not predict the function of CD8+ cells and inhibition was seen in all 3 groups. In the HIV positive cohort, this DNA vaccine was unable to induce detectable HIV-specific immunity to the vaccine delivered by either route. The VIA indicates some preservation of immune function in this cohort with the ability to inhibit virus at the pre-vaccination time point. Importantly, the vaccine was found to be clinically safe in both trials.

Table of Contents

Acknowledgements…… 2
Abstract…… 3
List of Abbreviations…… 12

1. Introduction…… 15
1.1 The origins of HIV…… 18
1.1.1 Zoonotic transmission and the emergence of a pandemic…… 18
1.1.2 HIV clades…… 18
1.2 HIV structure and life cycle…… 19
1.3 HIV transmission…… 21
1.4 HIV infection: Natural history and the immune response…… 22
1.4.1 Acute HIV infection…… 22
1.4.2 Genetic control of HIV set point and other pathogenic factors…… 23
1.4.3 The innate immune response…… 24
1.4.4 The antibody response…… 26
1.4.5 The T cell response…… 27
1.5 Chronic HIV and initiation or antiretroviral therapy…… 31
1.6 HIV prevention: preventative vaccines…… 32
1.6.1 Early trials using ALVAC-HIV and AIDSVAX gp120…… 32
1.6.2 The STEP and Phambili studies…… 33
1.6.3 Extended analysis of the STEP and Phambili immunogenicity results…… 34
1.6.4 RV144…… 35
1.6.5 Beyond RV144…… 36
1.6.6 Other HIV prevention methods…… 39
1.7 Therapeutic vaccines for HIV-1…… 39
1.7.1 Therapeutic immunisation strategies…… 39
1.7.2 Dendritic cell vaccines…… 42
1.7.3 Tat therapeutic HIV vaccine…… 42
1.7.4 Therapeutic HIV antibodies…… 43
1.8 HIV cure…… 44
1.8.1 Sterilising cure…… 44
1.8.2 Functional cure…… 44
1.8.3 Combination approaches to HIV cure- role of the therapeutic vaccine…… 45
1.9 DNA vaccination in HIV prevention and therapeutics…… 47
1.9.1 Optimising uptake of DNA…… 47
1.9.2 The importance of the route of DNA delivery- targeting the skin…… 50
1.10 Thesis hypothesis and aims…… 52

2. Materials and Methods…… 53
2.1 CUTHIVAC 001: Study subjects and vaccine specifics…… 53
2.1.1 Study participants…… 53
2.1.2 Ethical approval…… 53
2.1.3 Vaccine candidate and administration…… 53
2.1.4 Reactogenicity and safety…… 56
2.1.5 Immunogenicity sampling schedule and PBMC processing…… 57
2.2 CUTHIVAC 001 Immunogenicity: T cell IFN-ɣ ELISpot assay…… 57
2.2.1 Reagents and buffers…… 57
2.2.2 IFN-ɣ ELISpot assay…… 59

2.2.3 Statistical analysis…… 60
2.2.4 Supporting work: Intracellular cytokine staining…….. 61
2.3 CUTHIVAC 001: Viral inhibition assay…….. 62
2.3.1 Reagents and buffers…… 62
2.3.2 Optimisation of the assay…….. 63
2.3.3 Viral inhibition assay…… 64
2.3.4 Statistical analysis…… 68
2.4 CUTHIVTHER 001: Study subjects and vaccine specifics…… 69
2.4.1 Study participants…… 69
2.4.2 Ethical approval…… 70
2.4.3 Vaccine candidate and administration…… 70
2.4.4 Reactogenicity and safety…… 71
2.4.5 Human leucocyte antigen…… 71
2.4.6 Immunogenicity sampling schedule and PBMC…… 71
2.5 CUTHIVTHER 001 IMMUNOGENICITY: T CELL IFN-ɣ ELISpot…… 71
2.5.1 Reagents and buffers…… 71
2.5.2 IFN-ɣ ELISpot assay…… 72
2.5.3 Statistical analysis…… 73
2.5.4 Supporting work: Intracellular cytokine staining…….. 74
2.6 CUTHIVTHER 001: Viral inhibition assay…… 74
2.6.1 Reagents and buffers…… 75
2.6.2 Maraviroc optimisation experiment…… 76
2.6.3 VIA methods to address inhibition of endogenous virus…… 77
2.6.4 VIA methods to address inhibition of exogenous virus…… 79
2.6.5 Statistical analysis…… 80

3. Results: Trial Data CUTHIVAC 001…… 81
3.1 Introduction…… 81
3.2 Results…… 82
3.2.1 Participant recruitment…… 82
3.2.2 Enrolment and participant flow…… 85
3.2.3 Safety and reactogenicity…… 89
3.2.4 Tolerability and acceptability…… 94
3.3 Discussion…… 96

4. Results: Immunogenicity Data CUTHIVAC 001…… 99
4.1 Section A: IFN-ɣ T cell ELISpots Introduction…… 99
4.2 Results: IFN-ɣ T cell ELISpots…… 101
4.2.1 Electroporation and the IFN-ɣ response at the primary end point.. 101
4.2.2 Individual group primary end point analysis…… 105
4.2.3 IFN-ɣ responses across interim time points…… 110
4.2.4 Breadth of the IFN-ɣ response…… 114
4.2.5 Duration of the IFN-ɣ response…… 117
4.3 Supporting work: Data analysis from ICS…… 120
4.3.1 Primary end point analysis (all)…… 120
4.3.2 CD4+ response at the primary end point…… 120
4.3.3 Mean pooled peptide response at the primary end point…… 121
4.3.3 Correlation between ELISpot and ICS data…… 121
4.4 Section B: Viral inhibition assay Introduction…… 139
4.5 Results: Viral inhibition assay…… 140
4.5.1 Primary end point data and cross clade inhibition…… 140

4.5.2 Primary end point analysis between groups..... 144
4.5.3 Correlation of VIA with IFN-ɣ T cell ELISpots..... 148
4.6 Discussion..... 150

5. Results: Trial Data CUTHIVTHER 001..... 155
5.1 Introduction..... 155
5.2 Results..... 157
5.2.1 Participant recruitment..... 157
5.2.2 Enrolment and participant flow..... 158
5.2.3 HIV related characteristics..... 163
5.2.4 Safety and reactogenicity..... 164
5.2.5 Tolerability and acceptability data..... 168
5.3 Discussion..... 169

6. Results: Immunogenicity Data CUTHIVTHER 001..... 172
6.1 Section A: IFN-ɣ T cell ELISpots Introduction..... 172
6.2 Results: IFN-ɣ T cell ELISpots..... 175
6.2.1 Variability of baseline responses Week 0..... 175
6.2.2 Primary end point analysis..... 176
6.2.3 The placebo group: primary end point analysis..... 185
6.2.4 The integrase peptide pool: primary end point analysis..... 185
6.2.5 Interim time points..... 187
6.2.6 Duration of the IFN-ɣ response..... 188
6.2.7 Comparison of IFN-ɣ responses: CUTHIVTHER week 0 and CUTHIVAC primary end point..... 190
6.2.8 Change in CD4+ count at the primary end point: FIT-06 comparisons..... 193
6.3 Supporting work: Data analysis from ICS..... 196
6.3.1 Primary end point analysis (all)..... 196
6.3.2 Primary end point analysis within each group..... 196
6.4 Section B: Viral inhibition assay Introduction..... 209
6.5 Results: Viral inhibition assay Part 1 (endogenous virus)..... 210
6.6 Results: Viral inhibition assay Part 2 (exogenous virus)..... 213
6.7 Discussion..... 222

7. Final Discussion..... 227

References..... 237
Appendix..... 252

List of Figures

Figure 1.1: Adults and children estimated to be living with HIV. UNAIDS global report

Figure 1.2 Phylogenetic tree showing HIV-1 groups

Figure 1.3: Schematic of a HIV mature virion

Figure 1.4: Schematic overview of HIV-1 replication cycle

Figure 1.5: Stages of acute HIV infection

Figure 1.6: The cytokine storm in acute HIV infection

Figure 1.7: Illustration to show how latent HIV infection is established in T cell reservoirs

Figure 1.8. Diagram to show multifunctions of CD8+ T cell mediated HIV response

Figure 1.9. Diagram to represent the 'kick and kill' approach using voronistat

Figure 2.1: Diagram of Ichor electroporation handheld device

Figure 2.2: Schematic of T cell ELISpot assay

Figure 2.3: Graphic demonstration of viral inhibition assay procedure

Figure 2.4: A typical p24 standard curve from the CUTHIVAC 001 trial data

Figure 2.5: Plate plan for Weeks 0, 14 and 20 denoting presence of integrase peptide pool

Figure 2.6: Maraviroc optimisation assay CUTHIVER 001: p24 concentration (pg/ml) at Day 13 and Day 20

Figure 2.7: Adapted VIA where no exogenous virus is added

Figure 2.8: Adapted VIA where Maraviroc is added throughout the assay to block endogenous virus production

Figure 3.1: Diagram to show the 3 vaccination time points at weeks 0, 4 and 12 CUTHIVTHER 001

Figure 3.2: Adverts for CUTHIVAC 001 recruitment- The Metro, Evening Standard advert, London Pride Guide 'The Scene' Guardian newspaper supplement

Figure 3.3: Pie chart showing reasons participants who were screened were excluded from the trial

Figure 3.5: Consort diagram CUTHIVAC 001

Figure 3.6: Systemic and local reactogenicity events from participant diary cards

Figure 3.7: Photographic examples of hyperpigmentation and hypopigmentation changes from TC procedure

Figure 3.8. Tolerability questionnaire for EP

Figure 3.9: Acceptability questionnaire for EP

Figure 4.1: IFN-ɣ response at the primary end point shown by each peptide pool and group

Figure 4.2: Group comparisons for each peptide pool at week 14

Figure 4.3: Stacked peptide pool response at week 14

Figure 4.4: Stacked peptide pool response at week 14 by group

Figure 4.5: ID+IM Before/After of IFN-ɣ response from week 0 to week 14

Figure 4.6: TC+IM Before/After graphs of IFN-ɣ response from week 0 to week 14

Figure 4.7: EP+IM Before/After graphs of IFN-ɣ response from week 0 to week 14

Figure 4.8: Scatter plot showing IFN-ɣ response in EP+IM group at week 14

Figure 4.9 A: Gag response shown across time for participant C025 in the ID+IM group. B: CTL response shown across time for C009 in the TC+IM group indicating a high baseline response at week 0

Figure 4.10: Mean pooled peptide response across time shown by group with arrows indicating the presence of an early *Gag* response at week 4

Figure 4.11: Breadth of the IFN-ɣ response to the peptide pools

Figure 4.12: Breadth of the response to peptide pools in the EP+IM group

Figure 4.13 Mean SFU/M PBMC shown across time by group out to week 20

Figure 4.14: Before-after graphs showing all participant IFN-ɣ response at week 0, 14 and 20

Figures 4.15-4.18: CD4+ IFN-ɣ, IL-2, TNF-α, CD154 ICS data showing HIV specific responses at week 14 comparing all 3 groups

Figures 4.19-4.24: CD8+ IFN-ɣ, IL-2, TNF-α, CD57, perforin, granzyme B ICS data showing HIV-specific responses at week 14 comparing all 3 groups

Figure 4.25: ICS CD4+ mean pooled peptide response at week 14

Figure 4.26: CD8+ mean pooled peptide response at week 14

Figures 4.27-4.29: Correlation analyses for the ID+IM, TC+IM and EP+IM groups at week 14 examining the relationship between the T cell ELISpot IFN-ɣ results and the ICS data for IFN-ɣ

Figure 4.30: Graph showing $\log^{10}$ inhibition of virus panel at week 14

Figure 4.31 A: Viral inhibition by group at week 14

Figure 4.32: Breadth of viral inhibition by group

Figures 4.33-4.35: Before-after plots for ID+IM, TC+IM and EP+IM groups for each individual virus showing week 0 and week 14 $\log^{10}$

Figure 4.36: Correlation analysis between IFN-ɣ T cell ELISpot data from week 14 and $\log^{10}$ viral inhibition at week 14

Figure 5.1: Vaccination schedule CUTHIVTHER 001

Figure 5.2: Graph to show cumulative accrual of participants to the CUTHIVTHER 001 trial

Figure 5.3: Consort diagram outlining participant flow in the 2 groups

Figure 5.4: Participant motivation for taking part in the CUTHIVTHER 001 trial

Figure 5.5 A: Bar chart to show general symptoms and local arm and leg symptoms documented from immunisation form, safety calls and participant diary cards

Figure 6.1: Scatter dot plot showing high baseline IFN-ɣ response at week 0 (pre vaccination) by group

Figure 6.2: Box and whiskers graphs to show pooled IFN-ɣ response from all groups at week 0 and 14

Figure 6.3: Group comparisons for each peptide pool at week 14

Figure 6.4: EP+IM group (active) Before/After graphs showing difference in magnitude of IFN-ɣ response from week 0 to week 14

Figure 6.5: TC+IM group (active) Before/After graphs showing difference in magnitude of IFN-ɣ response from week 0 to week 14

Figure 6.6: EP+IM (active) group positive responders by IFN-ɣ with HLA types indicated

Figure 6.7: TC+IM (active) group positive responders by IFN-ɣ with HLA types indicated

Figure 6.8: Positive responders by IFN-ɣ in placebo recipients

Figure 6.9: Before-after plots showing all data pooled (for both groups receiving active drug), and EP+IM (active) and TC+IM (active) groups at week 0 and week 14

Figure 6.10: Before-after plot to show integrase responders at week 0 and week 14 and breakdown by group

Figure 6.11: Bar chart showing mean SFU/M PBMC for all peptides pool together (excluding integrase)

Figure 6.12: Group comparisons showing no statistical difference in IFN-ɣ response between weeks 0 and 20 in any group

Figure 6.13: Group comparisons of the integrase peptide pool showing no statistical difference in IFN-ɣ response between weeks 0 and 20 in any group

Figure 6.14: Comparison of the IFN-ɣ response at baseline (week 0) in the CUTHIVTHER trial of HIV infected volunteers and the primary end point (week 14) response in the CUTHIVAC trial

Figure 6.15: Comparison of the magnitude of IFN-ɣ response at baseline (week 0) in the CUTHIVTHER trial of HIV infected volunteers and the primary end point (week 14) response in the CUTHIVAC trial

Figure 6.16. Scatter dot plots to show total CD4+ cell counts at week 0 and week 14 by group

Figures 6.17-6.20: CD4+ IFN-ɣ, IL-2, TNF-α, CD154 responses at week 14 showing peptide specific responses comparing all groups

Figures 6.21-6.26: CD8+ IFN-ɣ, TNF-α, CD154, IL-2, granzyme B, perforin responses at week 14 showing peptide specific responses comparing all groups

Figure 6.27: Before-after plot to show the participants with detectable endogenous virus at week 0

Figure 6.28: Bar charts to show the number of participants inhibiting each virus at week 0 and 14. The positive responders only are show by group.

Figure 6.29: Graphs to show difference in log^{10} inhibition between week 0 and week 14 in all viruses and groups

Figure 6.30: Graphs for the EP+IM (active) group to show log^{10} inhibition for each virus at week 0 and week 14

Figure 6.31: Graphs for the TC+IM (active) group to show log^{10} inhibition for each virus at week 0 and week 14

Figure 6.32: Graphs for the placebo group to show log^{10} inhibition for each virus at week 0 and week 14

Figure 6.33: Box and whiskers graphs to show between group comparisons of log^{10} inhibition for each virus at week 14 with placebo recipients included

Figure 6.34: Breadth of viruses inhibited shown on graph at weeks 0 and 14 by group with median and interquartile range displayed

List of Tables

Table 1.1: Risk of HIV transmission per exposure from a known HIV-positive individual not on ART

Table 1.2 Table to show therapeutic vaccine trials that have been completed or are currently in progress.

Table 2.1: Inclusion/exclusion criteria CUTHIVAC 001

Table 2.2: Vaccine schedule for CUTHIVAC 001 including groups, route of vaccination and cumulative doses of DNA.

Table 2.3: Immunogenicity sampling schedule for serum, PBMCs and mucosal samples: CUTHIVAC 001

Table 2.4: Panel of viruses used in the viral inhibition assay

Table 2.5. Inclusion/exclusion criteria CUTHIVTHER 001

Table 2.6: Vaccine schedule for CUTHIVTHER 001

Table 2.7: Optimisation results from the integrase peptide pool in 8 donor cones

Table 2.8: Subjects used in Maraviroc optimisation assay with demographic information available

Table 2.9: Modified panel of viruses for CUTHIVTHER 001

Table 3.1: Source of trial related enquiries and number of participant enquiries generated per source

Table 3.2: Table to highlight 1/3 of participants enrolled were attracted to the study through social media

Table 3.3: Participant demographics CUTHIVAC 001

Table 3.4: Summary of unsolicited adverse events by group and relationship to vaccine

Table 3.5: Summary of clinical safety data (solicited events) from participant diary cards

Table 4.1: Vaccine schedule for CUTHIVAC 001

Table 4.2: IFN-ɣ responses at week 14 shown by group and per peptide

Table 4.3: Individual responders at each time point with response to peptide pool shown

Table 4.4: Table to show the number of participants meeting criteria for viral inhibition

Table 4.5. Table to show the number of viruses and type of virus inhibited at week 14 by group and participant

Table 4.6: EP+IM group table to show the number of peptide pools responded to at week 14 in the T cell ELISpot assay and whether the same participants displayed viral inhibition

Table 5.1: Participant demographics CUTHIVTHER 001

Table 5.2: HIV related characteristics CUTHIVTHER 001

Table 5.3: Summary of solicited adverse events occurring over the entire trial by group

Table 5.4: Summary of unsolicited adverse events by group with relationship to vaccine shown

Table 5.5: Tolerability data for EP+IM active and placebo

Table 6.1: Vaccine schedule for CUTHIVTHER 001

Table 6.2: Summary of IFN-ɣ responses at week 0 (pre-vaccination) by peptide and group

Table 6.3: INF-ɣ responses at week 14 shown by group and per peptide

Table 6.4: Summary of responders at week 14 shown by group and to which peptide

Table 6.5: Individual responders at each time point with response to peptide pool shown

Table 6.6 Comparison of the IFN-ɣ response at baseline (week 0) in the CUTHIVTHER trial of HIV infected volunteers and the primary end point (week 14) response in the CUTHIVAC trial

Table 6.7: Mean CD4+ counts with standard deviations shown by group at week 0 and week 14

Table 6.8: Endogenous virus production measured by p24 concentration in 5/30 participants with log^{10} inhibition shown at week 0 and week 14

Table 6.9: Median number of viruses inhibited by group at weeks 0 and 14

List of Abbreviations

Ab	Antibody
Ad	Adenovirus
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody mediated cellular phagocytosis
ADCVI	Antibody dependent cell mediated virus inhibition
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
ART	Antiretroviral therapy
ARV	Antiretroviral
AST	Aspartate aminotransferase
ATI	Analytical treatment interruption
AZT	Zidovudine
BASHH	British association for Sexual health and HIV
BHIVA	British HIV Association
BMI	Body mass index
BNAb	Broadly neutralising antibodies
CCR5	Chemokine receptor 5
CDC	Centre for disease control
CEFT	Cytomegalovirus/Epstein Barr Virus/Influenza/Tetanus
CL3	Containment level 3
CMV	Cytomegalovirus
CSSS	Cyanoacrylate skin surface stripping
CTL	Cytotoxic T lymphocyte
CTL	Peptide pool containing epitopes of protease, RT and gp160
CXCR4	Chemokine receptor 4
CXCL10	Chemokine ligand 10
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
EIAV	Equine infectious anaemia virus
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunospot assay
ENV	Envelope protein
EP	Electroporation
FDA	Food and drug association
FIV	Feline immunodeficiency virus
Gag	Group antigen
GCP	Good clinical practice
gp	Glycoprotein
GTU	Gene transfer unit
GWAS	Genome-wide association study
HDACi	Histone deacetylase inhibitor
HepBsAg	Hepatitis B surface antigen

HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HTLV	Human T-cell lymphotropic virus
HVTN	HIV vaccine trials network
IAVI	International AIDS vaccine institute
ICS	Intracellular cytokine staining
ID	Intradermal
IFN-ɣ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IN	Integrase enzyme
INI	Integrase inhibitor
IQR	Interquartile range
IV	Intravenous
KIR	Killer immunoglobulin-like receptor
LC	Langerhans cell
LTNP	Long term non progressor
MHC	Major Histocompatibility Complex
MHRA	Medicines and healthcare products regulatory authority
MITT	Modified intention to treat
MOI	Multiplicity of infection
MRC	Medical research council
MSM	Men who have sex with men
MVA	Modified vaccinia Ankara
MVC	Maraviroc
NAbs	Neutralising antibodies
Nef	Negative regulatory factor
NHS	National Health Service
NK	Natural killer
NNT	Number needed to treat
NNRTI	Non-nucleoside/nucleotide reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
P5	Pox-protein public private partnership
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PCP	Pneumocystis jirovecii pneumonia
PCR	Polymerase chain reaction
PD	Programmed death
PEP	Post-exposure prophylaxis
PHA	Phytohaemagglutinin
PHI	Primary HIV infection
PI	Protease inhibitor
PR	Viral protease enzyme
PrEP	Pre-exposure prophylaxis
PTC	Post treatment controller
RNA	Ribonucleic acid
RT	Reverse transcriptase enzyme

SFU/M	Spot forming units per million
SHIV	Simian-human immunodeficiency virus
SIV	Simian immunodeficiency virus
SOP	Standard operating procedure
TC	Transcutaneous
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRV	Truvada
UN	United Nations
UPAI	Unprotected anal intercourse
UPVI	Unprotected vaginal intercourse
UV	Ultraviolet
VIA	Viral inhibition assay
VL	Viral load
VMMC	Voluntary male medical circumcision
WHO	World Health Organisation

Chapter 1: Introduction

Between 1980 and 1981 four previously healthy men who reported to have sex with men (MSM) unexpectedly contracted community acquired *Pneumocystis carinii* pneumonia (PCP; now known as *Pneumocystis jirovecii* pneumonia) in Los Angeles. These patients also had multiple viral infections, including cytomegalovirus (CMV), and in three of these men periods of prolonged fevers of unknown origin. None of these men knew each other, nor had common contacts [1]. Further cases evolving during this short time period with additional cases from New York and San Francisco, prompted evaluation by the Centre for Disease Control (CDC) and the development of a case definition for national surveillance [2].

The link between these patients was the severity of immunosuppression witnessed with profound depression of absolute lymphocyte and T cell counts, whilst humoral immunity remained relatively intact [3]. This initial edition of Morbidity and Mortality Weekly Report (MMWR) marked the first official reporting of what would later become the global AIDS epidemic. Several cases followed amongst other MSM and injecting drug users with the addition of other clusters of rare diseases amongst gay men including the unusually aggressive rare cancer Kaposi's sarcoma. It was not until two years later when the virus causing this immunodeficiency was identified and named as Human Immunodeficiency Virus (HIV), the cause of Acquired Immunodeficiency Syndrome (AIDS) [4].

Initially HIV was thought to be linked to the Human T-cell lymphotropic viruses (HTLV) I and II, which were recently discovered human retroviruses [5]. However, it soon became apparent HIV had fundamental differences from the HTLV viruses including a greater degree of inter-strain variation and infectiousness. By 1984 firm evidence linking this virus to AIDS was established through advances in tissue culture and serological tests enabling US and French scientists to prove this virus was present in patients with AIDS and related diseases, with negative results in a large number of controls [6].

Over the last 30 years HIV has transformed from an untreatable fatal infection to a treatable condition susceptible to over 30 antiretroviral agents. In 1986 the first antiretroviral (ARV) agent zidovudine (AZT) was established and rolled out in a randomised controlled trial, acting as a nucleoside reverse transcriptase inhibitor on a critical point in the HIV life cycle and showing a significant survival advantage compared to placebo [7]. Although this was hailed as remarkable progress at the time, several limitations ensued including the development of drug resistance in some patients and severe toxicities such as life threatening anaemia. The development of this initial

drug however paved the way to the development of other classes of ARVs, including non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and more recently integrase inhibitors (INIs), transforming the trajectory of AIDS deaths worldwide [8].

To date 36.9 million globally are living with HIV, with 2 million new infections last year (Figure 1.1) [9]. By mid-2016 18.2 million people living with HIV were estimated to be receiving ART worldwide corresponding to an estimated 40% of all people living with HIV on ART [10]. Although globally new HIV infections have fallen by approximately 35% since 2000, in key areas such as Eastern Europe and Central Asia conservative estimates state new infections are rising exponentially by 30% over the same time frame.

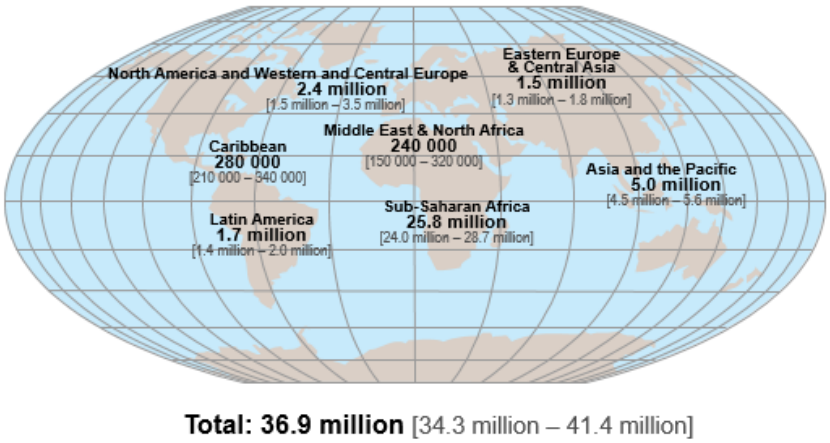


Figure 1.1: Adults and children estimated to be living with HIV. UNAIDS global report, 2014.

Key populations affected worldwide are MSM, transgender people, sex workers and injecting drug users. In the UK in 2015 over 101,200 people were living with HIV of whom approximately 70,000 were men and 30,000 women, with an estimated 47,000 men acquiring their infection through sex with other men [11]. Despite a decline in diagnosed HIV infections amongst MSM, there is still ongoing HIV transmission amongst those unaware of their status. As well as MSM, Black African men and women remain a key population at risk of HIV infection in the UK, therefore improving HIV testing and reducing late diagnoses remains a public health priority.

This MD(Res) project will focus on the immune responses to different routes of delivery of an HIV-1 DNA vaccine candidate. An effective prophylactic HIV vaccine could be a key prevention tool, which

alone or in combination with other HIV prevention measures may confer a significant impact on the HIV incidence.

Although ART has revolutionised life expectancy and morbidity in people living with HIV, ART alone cannot cure infection, a consequence of an inaccessible pool of latently infected cells. A therapeutic HIV vaccination approach could play a critical role in augmenting HIV-specific immune function in those already infected, with a potential role in HIV cure.

1.1 THE ORIGINS OF HIV

1.1.1 Zoonotic transmission and the emergence of a pandemic

There are two major strains of HIV infection; HIV-1 which is responsible for the widespread global pandemic, and HIV-2 thought to be genetically distinct from HIV-1, less infectious and more common in certain geographical areas such as West Africa [12]. This thesis will focus on HIV-1 unless stated otherwise.

Much progress has been made on disentangling the chain of events leading to the emergence of the HIV epidemic. Through complex molecular techniques, the source of the main group HIV-1 virus (group M) has been traced back to the *Pan troglodytes troglodytes* chimpanzee of central Africa [13], [14], and it is thought zoonotic transmission may have occurred as far back as the 1930s through the hunting and butchering of chimpanzee meat. Three other groups have also been identified (O, N and P), likely resulting from different cross species transmission events. Since the emergence of the epidemic, many questions have been raised such as how isolated zoonotic transmission may have led to a global pandemic, and through the exclusion of many different theories, two factors are thought to be linked to worldwide infections: urbanisation and healthcare [15].

It is now widely accepted that the dramatic rate of spread of HIV-1 was facilitated by improvements in transport links in the Congo and other central African countries. Transmission was facilitated by men leaving home for work and outnumbering women in smaller cities, encouraging the establishment of the sex trade. In addition and in particular in the Congo, programmes had been set up to attempt to treat various infectious diseases such as sleeping sickness and malaria. These infections at the time were treated with intravenous antibiotics. Unfortunately with poor health and sanitation precautions taken and a finite supply of equipment, syringes were reported to be reused several times accelerating the spread of not only HIV but also other blood borne infections such as hepatitis C [16], [17]. The virus was then thought to be exported out of African countries primarily to the United States (likely via Haiti) and onwards to Europe and other countries.

1.1.2 HIV clades

Within the HIV-M group mentioned above, there are further subdivisions into 10 recognized subtypes or clades A-K. These subtypes represent the genetic evolution of HIV within humans. According to recent studies the most prevalent HIV clades globally are A, B and C (Figure 1.2), and can be linked to specific geographical regions. This distribution pattern may be a consequence of the 'founder effect,' which refers to the ability to trace back local epidemics to a single variant of the

virus [18], or a more prevalent route of transmission resulting in the dominance of a particular subtype [19].

Subtype A is mostly linked to Central and East Africa plus Eastern European countries, Sub type B forms the most dominant subtype in Europe, Australia and America, and Subtype C mostly sub-Saharan Africa and India. The genetic diversity of HIV is an important factor which has impeded development of an effective vaccine and will be discussed in detail later.

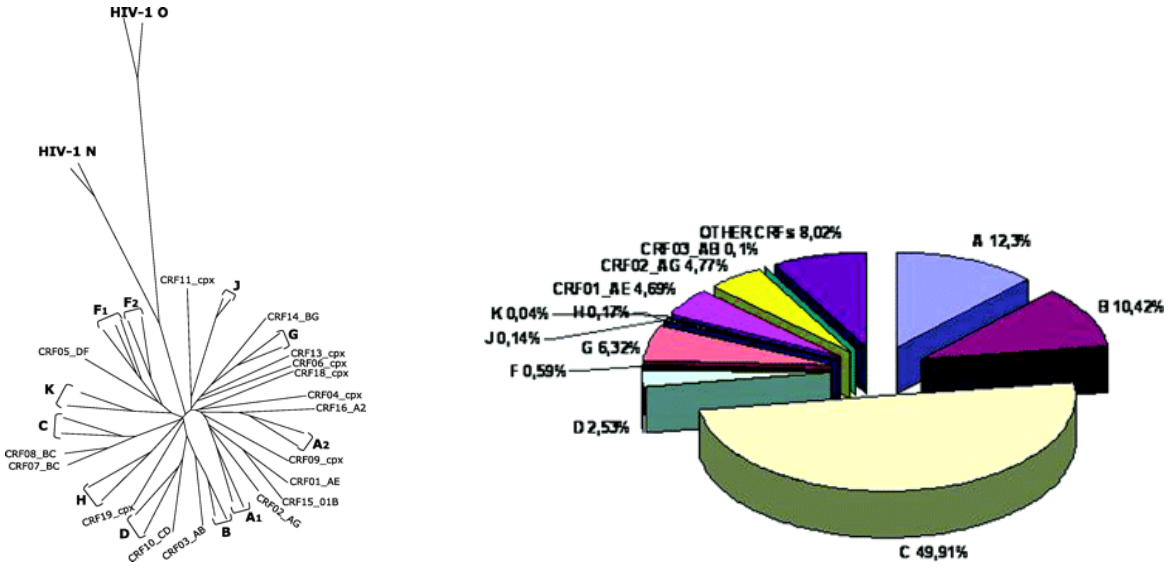


Figure 1.2: Phylogenetic tree showing HIV-1 groups M, O and N and subtypes within group M (left). Global prevalence of HV-1 genetic forms from group M only (right). *Adapted from Buonaguro et al. J of Virol 2007; 81:10209-10219*

1.2 HIV STRUCTURE AND LIFE CYCLE

HIV is a retrovirus belonging to the Lentivirus genus. These viruses are classically characterised as slow viruses with long incubation periods. Lentiviruses are unique in that they are able to infect both dividing and non-dividing cells [20]. Other examples of lentiviruses include simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV) and equine infectious anaemia virus (EIAV). Both HIV-1 and HIV-2 are closely related to SIV.

Like all retroviruses, the genome of HIV is encoded by RNA, and requires a host cell in order to replicate. An HIV particle has a tough viral envelope consisting of a lipid bilayer membrane surrounding the dense nucleocapsid core (Figure 1.3). This capsid contains the genomic RNA

molecules, the viral protease (PR), reverse transcriptase (RT), integrase (IN) enzymes and other cellular factors [21].

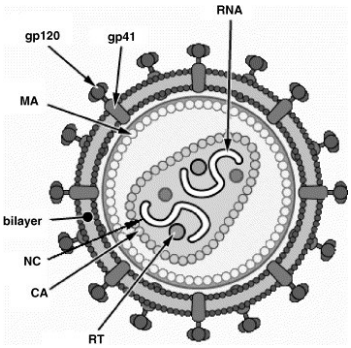


Figure 1.3: Schematic of a HIV mature virion. Reproduced from Sierra et al. Basic Virology of HIV-1 and its replication. Journal of Clinical Virology 2005:34;233-244

It is the HIV envelope protein (*Env*) which mediates viral entry into target CD4+ cells through fusion of viral and cell membranes. The *Env* polypeptide chain is initially a precursor gp160, which then is cleaved into two further fragments: gp120 and gp41. Gp120 directs CD4+ cell recognition and interaction with the CD4+ cell-surface receptor and co-receptors, leaving the gp41 subunit to promote fusion of viral and cellular membranes [22].

First, the viral gp120 binds to a CD4+ cell, which causes a conformational change in gp120, and results in co-receptors being exposed. This co-receptor is a necessary step for viral entry and establishment of infection. Depending on viral tropism either the co-receptor CXCR4 or CCR5 is engaged. The resulting change in gp120 triggers a corresponding change in gp41, allowing fusion of the viral and host cell membranes, resulting in the release of viral contents into the host cell [23].

Once the viral contents are released into the host cell, the viral reverse transcriptase enzyme converts viral RNA into DNA which is then integrated into the host genome involving the integrase enzyme. Because the reverse transcriptase enzyme is key for viral replication, it has been historically one of the most popular antiviral drug targets [24]. Next the production of viral proteins and assembly of new virions takes place at the cell surface. HIV protease enzymes cleave chains of proteins into individual functional HIV proteins and enzymes resulting in viral particles budding through the host cell membrane [25] (Figure 1.4). Finally, viral particles mature and go on to infect further CD4+ cells.

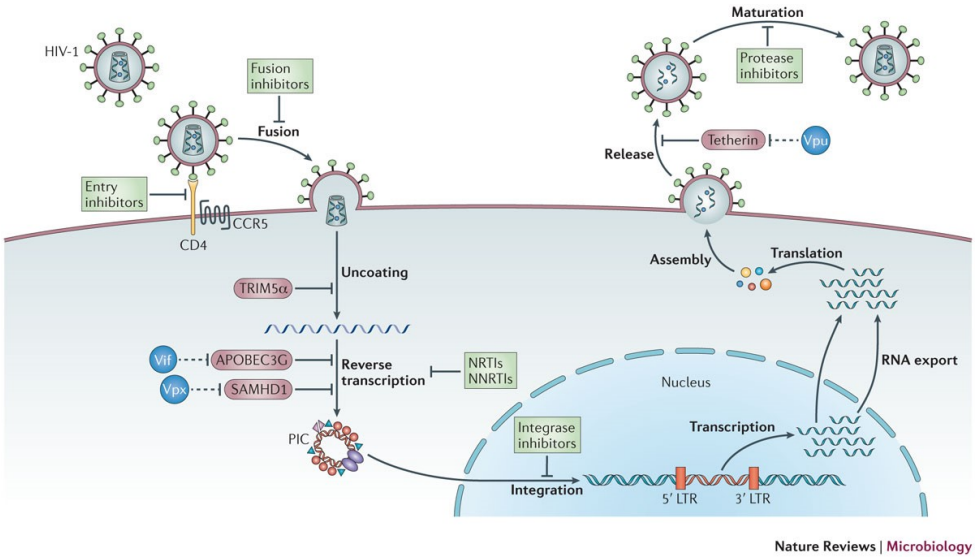


Figure 1.4: Schematic overview of HIV-1 replication cycle. Reproduced from Barre-Sinoussi et al. Past, present and future: 30 years of HIV research. Nature Reviews Microbiology. 2013:11;877-883

1.3 HIV TRANSMISSION

HIV can be transmitted through sexual contact, by sharing injecting equipment, by receiving blood transfusions or blood products from an infected person or from mother to child (vertical transmission). For sexual transmission to happen virus has to be present in a sufficient amount within the genital tract, the virus has to breach mucosal barriers, and finally virus has to make is way to susceptible cells.

When HIV is passed through sexual contact, the virus enters through the mucosal membranes of the rectum, vagina, cervix and urethra. Estimating the risk of HIV transmission per sex act however is complex and depends on both biological factors such as HIV viral load in the body fluid, and host factors including genetic and adaptive defences at the mucosal surface. The risk of an individual acquiring HIV following an exposure from a known HIV positive individual not on antiretroviral therapy (ART) is summarized below [26] (Table 1.1).

In the UK, the majority of HIV transmission is through condomless sex with the main risk groups being MSM and Black African men and women. According to data from the annual HIV report from Public Health England, there has been a continued rise in the number of MSM newly diagnosed with HIV up until 2015 (3360 new diagnoses compared to 2860 in 2010), but new diagnoses acquired through heterosexual sex over the same time period has declined, largely thought to be due to a reduction in diagnoses amongst black African men and women. The report also showed a consistent

low minority of people acquiring their infection through sharing injecting drug equipment or vertical transmission [27]; a consequence of free needle exchange programs for injecting drug users, and universal HIV screening in antenatal settings.

Table 1.1: Risk of HIV transmission per exposure from a known HIV-positive individual not on ART. Reproduced from the UK guideline for the use of HIV post exposure prophylaxis following sexual exposure (PEPSE), BASHH 2015.

Type of exposure	Estimated risk of HIV transmission per exposure from a known HIV-positive individual not on ART
Receptive anal intercourse	1 in 90
Receptive anal intercourse with ejaculation	1 in 65
Receptive anal intercourse no ejaculation	1 in 170
Insertive anal intercourse	1 in 666
Insertive anal intercourse not circumcised	1 in 161
Insertive anal intercourse and circumcised	1 in 909
Receptive vaginal intercourse	1 in 1000
Insertive vaginal intercourse	1 in 1,219
Semen splash to eye	<1 in 10,000
Receptive oral sex (giving fellatio)	< 1 in 10,000
Insertive oral sex (receiving fellatio)	< 1 in 10,000
Blood transfusion (one unit)	1 in 1
Needlestick injury	1 in 333
Sharing injecting equipment (includes chemsex)	1 in 149
Human bite	< 1 in 10,000

1.4 HIV INFECTION: NATURAL HISTORY AND THE IMMUNE RESPONSE

1.4.1 Acute HIV infection

The natural course of untreated HIV infection can vary widely with groups of individuals showing distinct patterns of disease progression. Acute HIV infection has been described by Fiebig et al, characterising the serial development of a measureable HIV-specific antibody response, using stored plasma samples from seroconverting plasma donors [28]. This classification describes the acute viraemia and early seroconversion defined by rapid viral replication and evolving antibody responses (Figure 1.5). After approximately day 7 following viral acquisition, in Fiebig stage II, viral gag protein, p24 antigen can be detected in the blood. During this phase an acutely infected individual may experience signs or symptoms associated with the classic seroconversion syndrome, although symptomatic HIV seroconversion is not universally present. These may include fever, rash, drenching

night sweats, fatigue, pharyngitis, diarrhoea and arthralgia, and typically presents and can last for up to 2-6 weeks after infection, coinciding with peak viraemia.

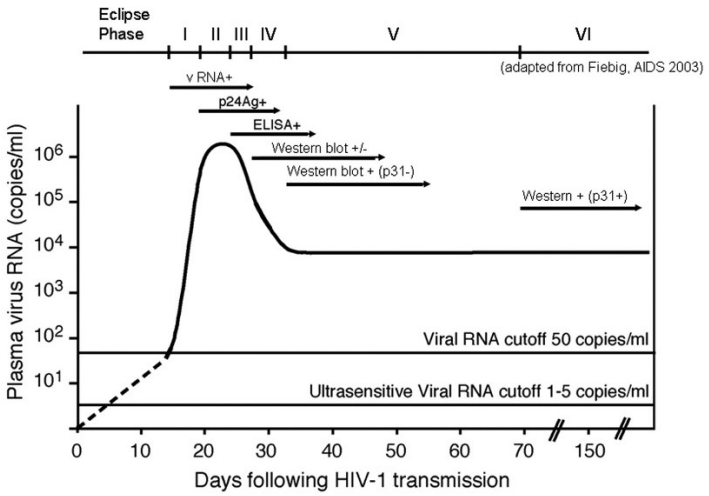


Figure 1.5: Stages of acute HIV infection as characterized by detection of viral particles and evolving antibody responses. Adapted from Fiebig et al AIDS, 2003.

In Fiebig stage III antibody levels may be sufficient to be detected with sensitive enzyme immunoassays (EIAs) and typically occurs 2 weeks after the onset of acute symptoms. Stages IV and V represent the evolution of western blot tests from indeterminate initially to positive with the final stage VI occurring 2-3 months after infection. The detection of acute HIV infection is challenging as few individuals present and are diagnosed during this short time period, but is thought to be critical to understanding and developing both prevention and cure strategies [29].

Approximately six months after HIV infection, plasma viraemia reaches a steady state known as the viral load 'set point.' This viral load (VL) set point is closely associated with disease progression in the absence of antiretroviral therapy and can be markedly variable. Several immune factors play a key role in establishing VL set point and will be discussed in the immunology section.

1.4.2 Genetic control of HIV set point and other pathogenic factors

As well as immune factors, there is some evidence to suggest host genetic factors may have an impact on VL set point and disease progression namely human leucocyte antigen (HLA) genotypes and the 32 base pair deletion in the CCR5 chemokine receptor. The first HIV genome-wide association study (GWAS) confirmed a major effect of HLA class I as a determinant of disease progression, in particular HLA B*5701 on lowering viral load and slowing CD4+ cell decline [30]. A further extensive genome study in over 2000 Caucasian HIV positive patients confirmed HLA B*5701

as the major contributor to control of viral set point, but also other independent genetic contributors to HIV control in the major histocompatibility complex (MHC) [31].

1.4.3 The innate immune response

The optimal immune response to any invading pathogen includes both innate immunity which mediates initial protection against infections, and the slower development of an adaptive immunity responsible for specificity, diversity and memory [32].

Once HIV has breached the mucosal barrier, viral interactions with the innate immune system determine whether the transmitted virus is eliminated at that site, or is able to disseminate. Innate responses are activated very rapidly and HIV must overcome these initial defences, but a secondary problem is lack of available and activated CD4+ cells to infect. HIV preferentially infects activated cells and to facilitate replication stimulates mucosal and subsequently lymphoid tissue dendritic cells (DCs) and toll like receptors (TLRs) to promote production of inflammatory cytokines, all of which act to recruit CD4+ cells thereby increasing the pool of available targets [33]. In addition, the virus is able to recruit DCs and bind to them, enabling direct invasion into the CD4+ cell which the DCs interact with in a process known as 'transfection' [34], [35].

Insight into the early innate defences has come from studying HIV uninfected plasma donors who subsequently acquired HIV. Studies showed initial responses include a dramatic increase in the levels of some acute phase proteins coinciding with cytokine release, predominantly interleukin 15 (IL-15), and interferon alpha (IFN-α) and a rapid increase in plasma viraemia. As plasma viraemia continues to increase there is a further 'wave' of cytokine release, namely tumour necrosis factor (TNF), IL-18, and chemokine ligand 10 (CXCL10) [36]. This 'cytokine storm' (Figure 1.6) is notably greater than that seen during other viral infections such as hepatitis B and C, indicating the magnitude may not be an essential part of viral clearance and in HIV may actually help propagate viral replication [37].

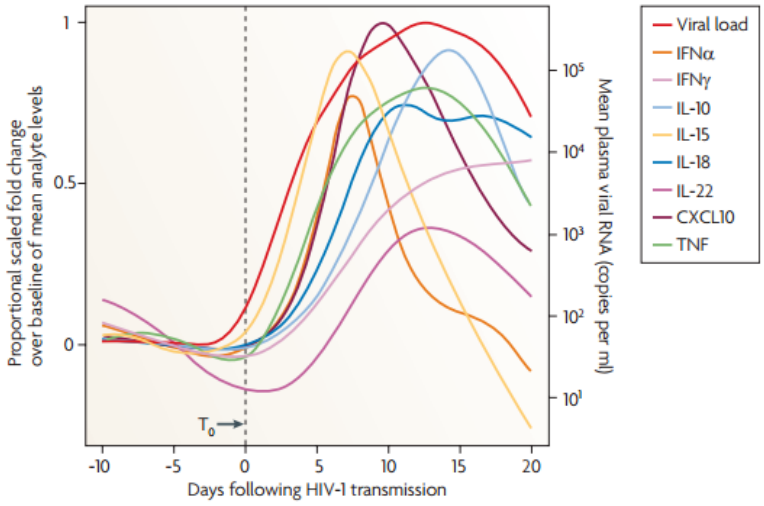


Figure 1.6: The cytokine storm in acute HIV infection showing the relative elevation of acute phase proteins and cytokines in relation to mean plasma viral load. Adapted from McMichael et al. *Nat Rev Immunol* 2010,**10**:11-23.

As with most viral infections, natural killer (NK) cells also become activated during acute infection, by the release of IL-2, 12 and 15 and this activation may be further be promoted by the direct recognition of pathogen associated molecular patterns (PAMPs). NK cells can recognize and kill infected cells through a number of different mechanisms. NK cells can express several activating receptors including NKG2D which can sense stress ligands on the surface of infected cells, and it has been suggested they can also directly sense viral peptides on infected cells [38]. In addition, the discovery of killer immunoglobulin-like receptors (KIRs) and their critical role in determining NK function through interactions with HLA class I molecules can act to inhibit or upregulate NK activity against HIV infected cells [39].

NK cells also play a key role in immune regulation of the acquired immune system through regulating DCs and the function of T cells, although these mechanisms are not yet fully understood [40]. This can be through direct NK cell mediated innate control of antigen load, or indirectly through interactions with APCs which can then modulate T cell activation. In mice, NK cells were shown to produce IFN-ɣ following exposure to MHC Class I tumour cells which led to activation of DCs and production of IL-12, which in turn enhanced CD8+ T cell mediated tumour control [41].

In summary, the innate immune response forms a key role in initial modulation of HIV infection through the activation of cytokines and chemokines, other key players such as NK cells and direct and indirect shaping of the acquired immune response.

1.4.4 The Antibody Response

Antibodies (Abs) are synthesized and secreted by B lymphocytes in the bone marrow and lymph nodes, and these B lymphocytes are the only cells capable of producing antibodies. Abs activate complement, influence effector cells such as NK cells, and activate other cells to encourage phagocytosis [42].

HIV Env antigens

The humoral or Ab response in HIV has been extensively investigated since the virus was discovered in 1983. The initial B cell response in HIV is to *Env* and can be detected within a week of infection. Much research has been conducted on the structure of *Env* namely gp120 and gp41, with multiple *Env* epitopes defined. Gp120 and gp41 are assembled into a trimeric complex that mediates virus entry into target cells, and HIV entry depends on the interaction of gp120 with receptors on the cell, CD4 and chemokine receptors as outlined previously [43].

Broadly speaking, Ab responses to envelope fall into 2 groups: non-neutralising, and neutralisation against a broad spectrum of HIV strains.

Non-neutralising Ab responses

Abs can respond to envelope but may not neutralise by typically binding to *Env* epitopes that are not presented on the 'functional' or trimer spike which mediates entry into the cells. However, it is now evident some non-neutralising Abs can still exhibit antiviral effect predominantly through Fc-mediated effector activity [44]. Immunoglobulin G (IgG) can interact with Fc receptors and trigger antibody dependent cellular cytotoxicity (ADCC) and Ab mediated cellular phagocytosis (ADCP), both of which have been demonstrated in vitro [45]. ADCC occurs via NK effector cells, whereas ADCP is mediated by the action of macrophages, monocytes or dendritic cells. In addition, in vitro Ab-dependent cell mediated virus inhibition (ADCVI) is also thought to provide a way of measuring the combined antiviral effects of Ab inhibitory mechanisms [46].

Interest in non-neutralising Abs has been predominantly driven by results of the RV144 Thai trial, which showed 31% protective effect in those who received the vaccine in the absence of neutralisation [47]. In this trial non-neutralising Abs have been associated with the V1V2 regions of gp120 with a lower risk of infection, and *in-vitro* these Abs have been shown to mediate ADCC [48]. Efforts to understand the interesting results of this trial are ongoing and the trial will be discussed in more detail in a later section.

Broadly neutralising Ab responses

Much effort has gone into understanding and characterising epitopes on *Env* proteins targeted by broadly neutralising Abs that develop during natural infection. High titres of broadly neutralising Abs (bNAbs) have been found in some chronically infected patients, with data from some studies indicating between 10-25% of patients display capacity to neutralise diverse HIV strains [49],[50]. Although these bNAbs may not directly protect infected patients, they can exert selective pressure on the virus.

As discussed, once an individual is infected, strong Ab responses develop against *Env*, and within a week of detectable viraemia, antibodies are directed specifically against gp41, gp120 and to some regions at the CD4+ binding site. Neutralising Abs can be detectable after a few months post infection, but this neutralisation activity targets autologous virus, which continues to evolve and escape the action of these antibodies [51],[52]. Most data has indicated bNAbs develop much later and often up to 2 years after infection [53]. The development of B cell amplification assays and single cell sorting has allowed much closer examination of individuals who display bNAbs, and one recent study has reported the presence of these antibodies within a year of infection- a potentially crucial piece of the puzzle as identification of these individuals with early bNAbs responses may help define new immunogen designs [54].

Several important lessons from animal studies have also been highlighted in recent years, with a range of broadly neutralising Abs showing evidence of sterilising immunity in macaques when challenged with chimeric SIV (SHIV) and in humanized mice models [55],[56]. One such important antibody is VRC01 which attaches to the CD4+ binding site and was examined in a rectal SHIV challenge model. Here VRC01 was first passively infused and followed by a viral challenge, with results highlighting important data on levels of Ab needed to be protective, allowing Phase I safety and pharmacokinetic studies to be developed and expanded [57],[58].

1.4.5 The T cell response

As discussed above, the neutralising antibody response in the majority of susceptible individuals is far too slow to prevent infection, or confer protection against disease progression. Although the T cell response is faster in comparison, a number of factors ultimately contribute to the inability to control viral replication.

CD4+ T cells

As a virus, HIV is unique in that it targets CD4+ cells which are critical in maintaining a healthy immune system. The infection and subsequent reduction in CD4+ T cells is the first important even in the pathogenesis of HIV infection [59]. As the virus multiplies rapidly in the first few weeks, there is a considerable reduction in CD4+ T cell counts in the acute stage, and over the long term, CD4+ cell death outweighs output of new cells from the thymus resulting in progressive depletion over time. Therefore preservation of HIV-specific CD4+ cells is vital for slowing progression of disease [60]. Pre-clinical data has shown in SIV-infected macaques loss of CD4+ cells correlates directly with worsening disease progression, possibly suggesting these cells may play a more active role in infection defence rather than a passive target for infection [61].

In humans the depletion in CD4+ count not only leaves the host immune system open to opportunistic infection, but evidence shows a subset of resting memory CD4+ cells which are not initially targeted by the HIV virus conceal latent HIV DNA which is impenetrable to antiretroviral therapy [62]. This latent pool is established by infected activated cells that become resting memory T cells [63] (Figure 1.7). The establishment of this latent viral reservoir is one of the key reasons why viral eradication remains such a challenge, and although very early treatment in primary HIV infection has been shown to decrease the size of the reservoir, treatment with ART alone is not able to eradicate latent virus [64],[65].

A relatively understudied aspect of CD4+ T cell function has suggested an early CD4+ T cell response to the virus results in CD4+ cell expansion and release of perforin and granzyme B, both of which are associated with control of viraemia [66]. Although this function of CD4+ cells is not widely accepted, it may suggest a more direct cytolytic action of CD4+ cells *in vivo,* important in early control of HIV infection.

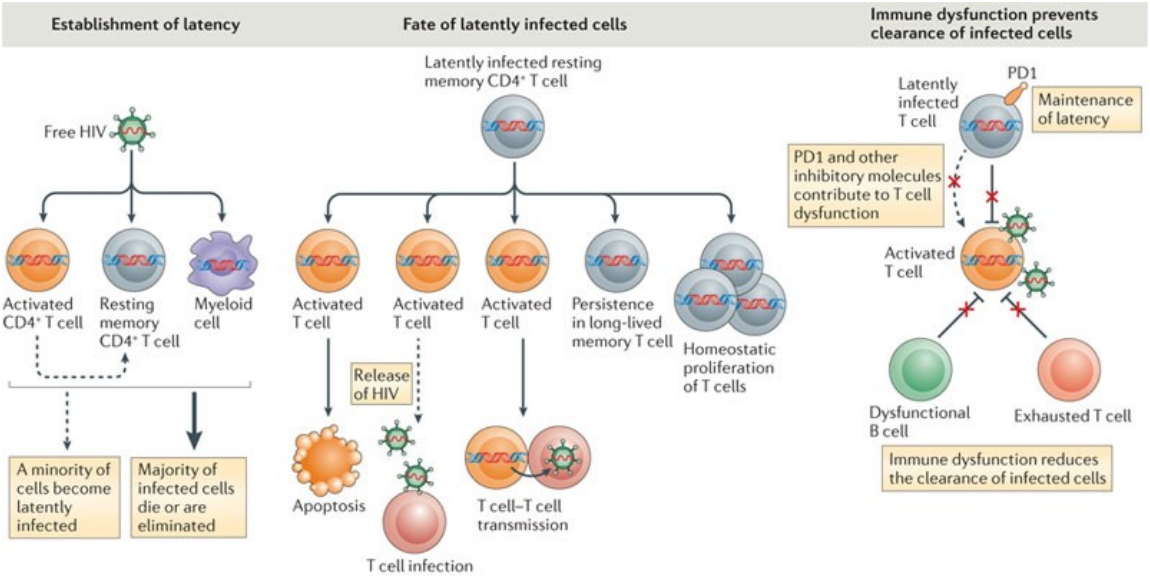


Figure 1.7: Illustration to show how latent HIV infection is established in T cell reservoirs. A small proportion of infected activated memory CD4+ cells revert to a resting state and either slowly die or become a source of new infections. Adapted from Deeks et al. Towards an HIV cure: a global scientific strategy. Nat Rev Immunol. 2012 July 20;12(8):607-14

CD8+ T cells

Antigen-specific CD8+ cells are a diverse population of cells with several important functions in disease control. In the context of HIV, these responses include production of cytokines and chemokines, cytolytic activity, and antigen-specific breakdown of MHC class I matched target cells (Figure 1.8) [67].

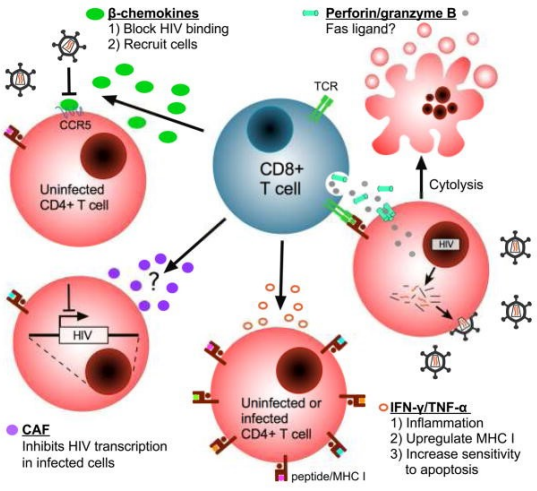


Figure 1.8. Diagram to show multifunctions of CD8+ T cell mediated HIV response. Adapted from Demers et al. CD8+ T cell effector function and transcriptional regulation during HIV pathogenesis. Imm Rev. 2013 July; 254(1):190-20

With respect to chemokines and cytokines, CD8+ cells trigger production of IFN-ɣ, a commonly use function to assess T cells responses to infection or vaccination. This cytokine in turn has been shown

to increase expression of other cytokines such as TNF-α which increases the susceptibility of virally infected cells to undergo apoptosis [68]. Perhaps the most important role of CD8+ cells however is to recognize and kill infected cells, predominantly through the secretion of granzymes and perforin [69]. Granzymes are proteases involved in the pathway that induces apoptosis in infected cells, and perforin is an important protein required for delivery of granzymes into the target cell [70],[71].

The role of CD8+ T cells in the control of viral set point has been discussed in a previous section and well documented in the literature [72]. However failure of the T cell response to completely control HIV replication is linked to narrow early HIV-specific CD8+ T cell responses that can only focus on a few viral epitopes, facilitating viral escape. Mathematical models have been used to show virus escape mutants are rapidly selected as viraemia falls from its peak in acute infection [73]. Although in those treated in early HIV infection escape mutations of common CD8+ cell epitopes may be rare [74], a recent study reported over 98% of proviruses in patients treated in chronic infection harboured escape mutations rendering the proviruses unrecognizable to CD8+ T cells [75]. In the SIV model these findings have been reciprocated, linking depletion of CD8+ cell depletion results to rapid progression of disease [76].

There is now mounting evidence from individuals able to control HIV in the absence of ART to suggest it may be the quality rather than the magnitude of the CD8+ response that is critical in controlling viral replication. Long term non-progressors (LTNPs) describes a cohort of individuals who are able to maintain high CD4+ counts (usually >500 cells/mm^3) for many years without therapy or progression of disease [77]. A subset of LTNPs termed 'elite controllers,' are able to maintain viral loads below the limits of detection (<50 copies) [78].

Although the exact mechanisms by which elite controllers control virus is not fully understood, HIV-specific CD8+ cells in controllers show a wider variety of 'polyfunctional' effector functions with superior cytotoxic CD8+ cells compared to progressors [79]. Data has shown in elite controllers CD8+ cells are able to upregulate perforin and granzyme B more easily than non-controllers [80], with a recent study linking the transcription factor T-bet present in higher numbers in elite controllers which positively correlates with perforin and granzyme B expression [81].

Finally T cell exhaustion can further lead to the inability to control viraemia. In untreated HIV infection as in many other chronic viral infections, persistent exposure to antigens can lead to the 'exhaustion' of CD4+ and CD8+ T cells [82]. This is thought to happen in a number of steps progressing through to effector dysfunction. In particular, exhausted T cells show impaired activation in relation to antigen and the reduced ability to produce multiple cytokines, followed in the late stages by

apoptosis of HIV-specific CD8+ cells. This state is associated with the activation of co-inhibitory molecules including programmed death 1 (PD-1) amongst others, which are thought to impair the anti-viral activity of CD8+ cells and further contribute to disease progression [83].

1.5 CHRONIC HIV AND INITIATION OF ANTIRETROVIRAL THERAPY

Once HIV has crossed the mucosal barrier and established itself, in the absence of ART there is thought to be a steady decline in total peripheral CD4+ T-cell counts of approximately 67 cells per year [84]. Once the CD4+ count has fallen below 200 cells/mm^3, there is a significant risk of opportunistic infections and malignancies due to damage to the immune system. Furthermore, late presentation of HIV at diagnosis (CD4+ cells <350), remains a significant problem in the UK with an estimated 39% of adults diagnosed late from UK data in 2015 [11]. Late diagnosis is linked with poorer outcomes, higher rates of morbidity, longer hospital stays and premature death [85]. With declining CD4+ counts opportunistic and AIDs-defining illnesses including *Pneumocystis jirovecii* pneumonia, *Mycobacterium avium* complex, *Cryptococcal meningitis* amongst others are seen which can cause both significant morbidity and death. Without treatment these AIDS-defining illnesses are invariably fatal.

Since the advent of ART and the use of prophylactic antibiotics however, the incidence of these classic opportunistic infections has dramatically declined [86]. Worldwide treatment guidelines have differed on when to start ART, and in the UK up until last year the British HIV Association (BHIVA) recommended starting therapy when CD4+ levels had dropped to below 350 cells/mm^3. More recently however, data from the START study has recommended treatment for everyone regardless of CD4+ count.

The START study was the first randomised controlled trial to provide evidence for initiation of ART at higher CD4+ counts, as prior to this data available was from observational studies only [87],[84]. Over 4500 HIV positive patients with CD4+ counts over 500 cells/mm^3 who had not yet started ART were randomised to either start therapy immediately at the higher CD4+ counts, or to start once their CD4+ count had dropped below 350 cells/mm^3 (or the development of an AIDS-related event or other event requiring initiation of ART) [88]. The primary endpoints in this study included the incidence of any serious AIDs-related event or non-AIDS related event (including myocardial infarction, stroke, renal and liver disease or non AIDs-defining cancers).

At the end of 3 years follow-up, the primary end points were reached in 42 patients in the immediate treatment group and 96 in the deferred treatment group with an estimated hazard ratio in the deferred treatment group for a serious AIDS-related event of 0.28 (p=<0.001), a serious non AIDS-related event of 0.61 (p=0.04), and 0.58 (p=0.13) for death from any case. The authors concluded immediate initiation of therapy was superior to deferral with benefits evident for both AIDS and non AIDS-related events. These findings have led to changes in WHO [89], international [90], and National HIV treatment guidelines [91], recommending initiation of ART regardless of CD4+ count, now with evidence not only of public health benefit by reducing the risk of transmission but also individual benefit.

1.6 HIV PREVENTION: PREVENTATIVE VACCINES

Despite the global need for an effective prophylactic vaccine, very few vaccine concepts have progressed to efficacy trials. The challenges include the nature of HIV as a highly variable and rapidly evolving virus which can integrate into the host genome, establish latency and evade both cellular and humoral host immunity [92]. Furthermore although some protective immune correlates have been identified in the trials below, particularly from RV144, the mechanisms that confer protection are still not fully understood and until they have been reproduced in further clinical trials (currently in progress) the mechanisms behind these correlates are yet to prove generalizable beyond RV144. The key preventative clinical trials are outlined below.

1.6.1 Early trials using ALVAC-HIV and AIDSVAX gp120

Initially a glycoprotein gp120 (AIDSVAX B/B) failed to show efficacy in two previously conducted clinical trials, both of which showed limited immunogenicity with no protective effect in MSM, at risk heterosexual women or injecting drugs users [93],[94]. This was followed by the development of the attenuated canarypox vector HIV vaccine which encodes HIV-1 gp120 (ALVAC-HIV vCP1452) consisting of the entire *Gag* protein, a portion of *pol* and several human CTL epitopes from *Nef* and *Pol*. In HVTN 203, 4 doses of this vaccine were administered at months 0, 3, and 6 alone or in combination with 2 different gp120 regimens (3 and 6 months vs 0, 1 and 6 months). The primary end points of this trial were to obtain an expanded safety profile and to investigate whether CD8+ responses could be detected at adequate frequencies to qualify the vaccine for Phase III trials and beyond [95]. This was also one of the earliest trials to use IFN-ɣ ELISpot assays to measure and quantify the CD8+ T cell response as a primary end point [96].

The trial enrolled 300 HIV-uninfected participants across 10 sites in the USA between December 2000 and August 2001. Results showed overall the vaccine regimen was safe and well tolerated with respect to pre-defined safety parameters. Immunogenicity results however were limited, with CD8+ T cell responses significantly lower than expected based on earlier generation canarypox products, and this particular combination was therefore not extended to Phase III.

In contrast to these trials, a Phase II study in Thailand of the ALVAC-HIV (vCP1521) vaccine boosted with a gp120 B/E (AIDSVAX B/E) enrolled 133 HIV-negative Thai adults aged 20-50, who were given ALVAC-HIV at weeks 0, 4, 12 and 24, with either 200µg or 600 µg AIDSVAX B/E at weeks 12 and 24 in a prime-boost regimen [97]. The combination was found to be safe and immunogenic, with lymphoproliferative responses to gp120 induced in 63% of volunteers, and HIV-specific CD8+ T cell responses in 24%, with antibody responses increasing in frequency and magnitude with the dosing level of AIDSVAX B/E. This trial met both adequate safety and immunogenicity parameters enabling advancement to Phase III; arguably the most cited HIV-1 vaccine trial to date (RV144).

1.6.2 The STEP and Phambili studies

Following on from the two AIDSVAX trials and prior to RV144, was the third large scale efficacy trial known as the STEP study or HVTN 502 [98]. STEP directly assessed the efficacy of a cell-mediated immunity vaccine to protect against HIV-1 using the MRKAd5 HIV-1 replication incompetent Ad5 vectors expressing *Gag, Pol,* and *Nef*, as these antigens were commonly recognised during natural infection and remained fairly conserved across different clades of HIV-1. In early trials this vaccine was shown to be safe and able to elicit immune responses in HIV uninfected participants regardless of prior Ad5 exposure [99].

This was a multicentre double-blind randomised placebo controlled trial across North America, South America, the Caribbean and Australia, which enrolled over 2500 at risk men and women to receive the vaccine or placebo at weeks 0, 4 and 26, with local and systemic reactogenicity assessed 2 weeks after the final vaccine. Of note, included in the trial were individuals with Ad5 antibody titres both less and greater than 200. The primary objectives were to assess safety and tolerability in those with Ad5 Ab titres <200 and the reduction in HIV acquisition or a decrease in HIV viral load (VL) set point in those infected at 3 months.

Unfortunately, at the first interim analysis the trial met 'futility' boundaries and was therefore stopped. Specifically, the HIV incidence in the modified intention to treat (MITT) population was 3.6% in men and 0.2% in woman, and slightly higher in vaccine than placebo recipients. In the expanded analysis, 49/914 male vaccinees became infected compared to 31/922 men receiving

placebo, with rates twice as high in those with Ad5 Ab titres greater than 18. Viral load set point results showed no difference between groups. The trial concluded this vaccine neither prevented nor lowered VL set point compared to placebo in those with low Ad5 Ab titres.

The interesting and perhaps surprising part of this trial was that despite generating reasonable IFN-ɣ ELISpot responses in most participants receiving the vaccine, this did not correlate with immune protection, raising the question of whether the magnitude, quality and specificity of the immune response generated by this vaccine was sufficient to control viral replication. The trial also raised the controversial question of whether repeated administration of the Ad5 vector may cause an ill-defined effect on the immune response leading to increased risk of HIV acquisition.

The Phambili study or HVTN 503 was designed and started soon after STEP using the same vaccine but aimed at assessing efficacy in a predominantly Clade C region of the world and in populations with high pre-existing immunity to Ad5 [100]. Five sites across South Africa recruited heterosexual men and women at risk of HIV, however enrolment and vaccinations were stopped soon after reports from the STEP study interim analysis were available.

Early analysis of the Phambili trial showed high T cell responses to *Gag* with no differences in HIV VL set point compared to placebo. Unlike the STEP study, there was no evidence of increased risk in vaccinated participants compared to placebo nor in Ad5 seropositive participants. However, it is thought perhaps the premature unblinding of the study may have resulted in limited conclusions.

1.6.3 Extended analysis of the STEP and Phambili immunogenicity results

An extended analysis of these 2 studies has raised the question of whether T cell based vaccines held promise as protective HIV immunogens, and why despite high T cell responses no effect was seen on HIV acquisition. Furthermore, there seemed to be an increased risk of infection in a subgroup of men who were both uncircumcised and had pre-existing Ad5 immunity compared to placebo.

One suggested hypothesis was in men with pre-existing Ad5 immunity, the vaccine activated vector-specific CD4+ T cell potentially resulting in increased immune activation and increased expression of CCR5 [101]. Furthermore using intracellular cytokine staining (ICS) investigators were able to show high IFN-ɣ responses after 2 vaccines, especially in those with low Ad5 Ab titre (<200), and this was not impaired in those with HIV infection [102]. These responses were higher in rate and magnitude than that reported for many of the candidate immunisation regimens tested over the past 15 years, discounting the possibility of a weakened vaccine product in these trials [103]. However, an important point here is that despite high CD8+ T cell responses, these were still lower than those found in long-

term non-progressor cohorts, suggesting perhaps the CD8+ responses generated in participants in these trials did not have sufficient breadth to recognise epitopes within transmitted viral strains [104].

Extended follow up of Phambili showed although there did seem to be increased HIV acquisition among vaccinated men. This was not associated with circumcision status of Ad5 status, generating different results to STEP and further raising questions on whether the different study population in the Phambili study (and with higher Ad5 seropositivity in an African cohort) may have contributed to this [105].

1.6.4 RV144

Despite these early trial disappointments and to the surprise of many investigators, the early AIDSVAX trials were viewed as safe and immunogenic paving the way to a Phase III trial which so far has been the only clinical trial to show any efficacy with respect to HIV acquisition.

RV144 set in Thailand was a community based, randomised double blind placebo-controlled efficacy trial with the focus on prevention of HIV infection and the effect of vaccination on early VL set point after infection [47]. Over 16,000 HIV uninfected participants were enrolled between September 2003 and December 2005, receiving ALVAC-HIV at weeks 0, 4, 12 and 24, with AIDSVAX B/E boosting at weeks 12 and 24. In total there were 125 new HIV infections (51 in the vaccine group, 74 in the placebo group) in the MITT, equating to a vaccine efficacy of 31.2%. As HIV testing was conducted at week 24, the authors deemed it was not possible to ascertain which dose of vaccine may have been associated with an early protective effect.

In terms of effect on VL set point, there were no differences in the vaccine group compared to placebo, and no differences in post-infection CD4+ counts. In addition, only 19.7% of volunteers were found to have HIV-specific T cell responses measured by IFN-ɣ when exposed to either *Env*, or *Gag* after the final vaccine. The authors concluded a modest protective effect on HIV acquisition in this study, and at that stage the immune mechanisms mediating these results were not fully understood, prompting further extensive analysis of the results.

In a follow up analysis, 41 vaccinated patients who had acquired HIV-1 during the trial were examined and compared to vaccinated uninfected controls as well as placebo recipients [48]. The main immunological findings pointed towards IgG binding to the V1V2 regions as an inverse correlation with risk of infection, and conversely IgA binding to *Env* was directly correlated with infection. This pointed towards further investigation into the potential roles of V1V2 and *Env*-specific IgA antibodies in the modulation of HIV infection.

1.6.5 Beyond RV144

Since RV144, a few other key trials have reported and either reached completion or been halted due to futility. HVTN 505 examined the DNA/rAd5 HIV-1 vaccine in men and transgender women in those with Ad5 titres <18 with similar end points to previous trials [106]. This trial was stopped prematurely after an interim analysis showed similar infection rates in the vaccine and placebo group (at week 28, 27 infections in vaccine group, 21 in placebo group, p=0.44). The trial concluded the vaccine regimen did not reduce either the rate of HIV-1 acquisition nor viral load set point in the population studied.

Evaluation of the RV144 regimen is now being undertaken in HIV uninfected South African adults (HVTN 097), in a similar regimen to ascertain whether immune response profiles in this population are similar to the Thai trial. This trial has not yet reached completion, but early results have suggested response rates and magnitudes of the *Env*-specific CD4+ T cells in South African adults were at least comparable if not better than those elicited in RV144 (unpublished data). In addition, the Pox-Protein Public Private Partnership (P5) has developed regimens for testing in follow-on trials to RV144 in both Southern Africa and Thailand, as well as recall of trial participants in RV144 with the aim of re-boosting immune responses. These trials are currently in progress and may provide some key progress in the preventative vaccine field.

1.6.6 Other HIV prevention methods

Antiretroviral therapy for the prevention of HIV transmission

HPTN 052 was a large study designed to investigate the reduction in risk of transmission from infected people to their sexual partners. Previous data from observational studies had demonstrated in serodiscordant couples ART can reduce the risk of sexual transmission of HIV in the uninfected partner [107], but as yet no randomised trial had shown this. This was the first randomised controlled trial to enrol 1763 serodiscordant couples across 13 sites in 9 countries. The couples were randomised to either receive ART at the time of enrolment for the positive partner (CD4+ count between 350-500 cells/mm^3) or delayed ART when the infected partner's CD4+ count dropped to less than 250 cells/mm^3 on two consecutive tests, or an AIDS defining illness developed [108]. Genetic linkage assays were used to ensure any new infections were linked to the index positive partner enrolled in the study and not other sexual partners.

An interim analysis which included data after a median follow up of 1.7 years revealed in the immediate ART arm there was a 96% lower risk of infection, with added health benefits to the index participants in starting early ART [109]. After this interim analysis all index patients were offered ART

regardless of CD4+ count. A final analysis after 5 years of follow up documented 19 new infections in the early ART group and 59 in the delayed ART group with viral linkage determined in 92% of these new infections. Those infections found to be linked and present in the early ART group (8 in total) were thought to have arisen prior to the index patient becoming virally suppressed on ART or infection had occurred after ART had failed in the index patient. The final results of the HPTN 052 trial support the importance of suppressing viraemia to prevent transmission, leading to the WHO adapting its guidelines to include recommendations for the provision of ART to all those living with HIV regardless of CD4+ count [89].

One major limitation of the HPTN 052 study was it enrolled predominantly heterosexual couples only, prompting further investigation on applicability to same-sex couples. The PARTNER study was a prospective observational study which enrolled 1166 serodiscordant couples including MSM where the HIV positive partner was taking ART and the couple reported condomless vaginal and anal sex [110]. This study was important as prior studies included predominantly heterosexual sex acts in the context of ongoing reported condom use, with no data available for condomless vaginal or anal sex when the HIV positive partner was suppressed on ART.

Results showed a total of 11 HIV negative partners (10 MSM, 1 heterosexual) acquired HIV infection over the course of this study, but none of these were phylogenetically linked transmissions. This translated to an estimated rate of transmission of zero through any condomless sex act when the positive partner was on ART with an HIV VL of <200 copies, although the study did conclude a longer follow up period may be necessary for more precise estimates of risk.

Post exposure prophylaxis (PEP) for HIV

Evidence for PEP following sexual exposure to HIV is largely from pathogenesis studies indicating a small window of opportunity to initiate ART following sexual exposure (estimated at a maximum of 72 hours) [111],[112]. Although randomised controlled trials for PEP are lacking, data from animal studies [113], retrospective data collected on occupational exposure to HIV [114] and data from the AIDS Clinical Trials Group 076 on vertical transmission and the use of AZT in neonates [115], have indicated a protective effect.

Factors influencing PEP include the viral load of the source patient if known, type of sex as an indicator of risk, and an estimation of HIV prevalence if the HIV status of the source patient is unknown. Although PEP is not considered as first line in HIV prevention, guidelines from the British Association for Sexual Health and HIV (BASHH) state it should be recommended where there is a

significant risk of HIV transmission, and forms the backbone of HIV prevention amongst those at risk in the UK [116].

Pre-exposure prophylaxis (PrEP) for HIV

Three large clinical trials have demonstrated the efficacy of the use of Truvada (TRV) as PrEP; iPREX, IPERGAY and the PROUD study. Evidence for daily dosing of Truvada comes from the PROUD study based in the UK where over 500 MSM reporting recent condomless anal sex were randomised to receive either immediate PrEP in the form of daily TRV or delayed PrEP after a period of 12 months (during which time PEP could still be accessed as well as risk reduction counselling and regular STI screening) [117]. An interim analysis revealed an unexpected and unacceptably high HIV incidence in the delayed PrEP arm (3 in the immediate group; 1.2/100 person years compared to 20 in the delayed group; 7.8/100 person years), with a relative risk reduction of 86%. In this trial the number of people needing to treat with PrEP to prevent one infection (NNT) was low at 13. Following the interim analysis all participants were offered daily PrEP with the findings strongly supporting the addition of PrEP to the standard of prevention for MSM at risk of HIV infection.

In the IPERGAY trial high risk MSM reporting recent condomless anal sex where randomised to receive either TDF-FTC or placebo, using an 'on demand' regimen. This consisted of a loading dose of 2 pills of TDF-FTC or placebo 2-24 hours before sex followed by a further pill 24 hours later and further 24 hours dosing until 48 hours after the last sex act [118]. This regimen proved to be highly protective against HIV translating to a relative reduction in risk of HIV infection of 86%. The number needed to treat (NNT) to prevent 1 HIV infection in this trial was 18, with most people taking on average 15 pills a month.

PrEP has been made available in several countries to date including the United States, Australia, France and most recently Kenya, but is currently challenged by resource limitations in most settings including the UK

Voluntary male medical circumcision (VMMC)

The efficacy of VMMC has been demonstrated in randomized clinical trials in Uganda, Kenya and South Africa [119],[120],[121]. Long term effectiveness has also been shown in a post-trial follow up in Uganda where amongst 4996 HIV-negative uncircumcised young men randomised to either receiving immediate circumcision or delayed circumcision after 24 months, an interim analysis showed efficacy in the immediate arm and therefore all participants were offered VMMC. Post-trial follow up of this cohort for 5 years showed HIV incidence to be 0.50/100 person years in circumcised men and

1.93/100 in uncircumcised men translating to an efficacy of 74% (p=<0.0001) in those circumcised [122].

VMMC now forms part of the United Nations Fast Track Strategy goals, with a target of 80% to be reached in high prevalence settings across sub-Saharan Africa by 2020 [123]. In addition mathematical models have shown even if the final '90' in the UN 90-90-90 target is not reached (90% of people on ART achieving viral suppression), the scale of up VMMC to 90% can lead to a reduction in HIV incidence to nearly the same levels as those achieved by ART alone, with considerable cost savings [124].

1.7 THERAPEUTIC VACCINES FOR HIV-1

Although the progress and development of ART has transformed HIV from a fatal disease to a treatable chronic illness particularly in resource rich settings, the sustainable delivery of lifelong ART cannot keep up with the rate of new infections. ART is able to suppress viral replication and prevent onward transmission, a key factor in the UNAIDS 90-90-90 target [125]. However, this is dependent on individual motivation to adhere to therapy for life, avoid drug-drug interactions, and for lifelong access to medication, not to mention profound economic strains on National Health Services. In addition, ART is unable to eliminate HIV-1 infection completely, with evidence of persistent viral replication in compartments and reservoirs impenetrable by even the most potent ARVs [126]. This ongoing viral replication also results in the inability of immune function to be restored which is associated with chronic immune activation and incomplete restoration of CD4+ T cells [127].

It is for these reasons growing attention has shifted towards exploring alternative approaches to limit or eradicate the HIV reservoir. Initially, therapeutic vaccines were tested to see if they could delay the need for ART [127], however now in the context of treatment for everyone this may no longer be entirely relevant. Newer approaches include the use of a therapeutic vaccine to augment ART therapy, reduce the size of the reservoir and contribute a role in HIV cure. Mechanisms for these important goals included eliciting potent anti-viral CD8+ T cells, CD4+ T cells and neutralizing antibodies to control viral replication [128].

1.7.1 Therapeutic immunisation strategies

No therapeutic vaccine has yet shown sufficient efficacy to warrant market approval, although a growing number of vaccines in clinical trials are currently under evaluation, a few of which are outlined in this section.

Since HIV therapeutic vaccine trials began, a major challenge has been how best to assess vaccine efficacy in this context. Many therapeutic vaccine trials use an analytical treatment interruption (ATI) where ART is stopped temporarily and various outcome measures are used to assess vaccine induced immune-mediated viral control [129], [130]. ATI can reflect the ability of the immune system to effectively control HIV replication, and allow for assessment of a potentially new viral load set point following treatment interruption, or changes in the size of the viral reservoir [131]. ATI however is not always appropriate and applicable to all therapeutic vaccine trials. Other trial outcomes include clinical end points such as changes in CD4+, CD4:CD8 ratio or the development of specific clinical events [132],[133].

Therapeutic vaccines based on the HIV-1 p24 protein have been use to assess whether in HIV infected individuals on effective ART, the vaccine could be immunogenic and decrease viral load after a period of treatment interruption. This trial which used the Vacc-4 peptide based vaccine showed no benefit to vaccination, as the proportion of participants having to restart ART before the end of the study was significantly high [134].

Relevant to my thesis, the FIT Biotech GTU®MultiHIV B clade vaccine based on six viral proteins has also shown promising results. In HIV-infected ART-naïve subtype C individuals in South Africa with relatively high CD4+ counts (>350 cells/mm^3), the vaccine was administered at weeks 0, 4 and 12 with a booster at week 76 and delivered either intradermally (ID) or intramuscularly (IM), and compared with placebo. The results of this trial showed the vaccine was safe and well tolerated, with a 0.5 decrease in log^{10} HIV-RNA (p=0.012) when administered IM compared to other groups, with moderate increases in CD4+ counts although these were not found to be statistically significant (p=0.066) [135]. This vaccine is used in the two clinical trials on which my MD(Res) thesis is based.

There are several other therapeutic vaccine trials currently in progress utilising concepts which include DNA-based vaccines incorporating viral vectors, RNA based vaccines and dendritic cell vaccines (Table 1.2), but few have so far advanced to Phase III [136]. These trials are mostly based on safety and immunogenicity and not efficacy, but have highlighted important advances in our understanding of the cellular response. In the next section I have outlined newer vaccine strategies with encouraging results, moving away from conventional therapeutic vaccination. Newer approaches for therapeutic vaccination concentrate on antigen selection and novel vaccine delivery systems.

Table 1.2 Table to show therapeutic vaccine trials that have been completed or are currently in progress. Adapted from Pantaleo & Levy. Therapeutic vaccines and immunological intervention in HIV infection: a paradigm change. Current Opinion in HIV and AIDs. 2016: 11(6); 576-584

Trial	Trial number	Sponsor	Phase	Status
DNA-based vaccine				
GTU-MultiHIV B Clade Vaccine	NCT02457689	Imperial College London	I/II	Active
DermaVir (topically applied DNA)	NCT00711230	Genetic Immunity	II	Unknown
DermaVir	NCT00918840	Genetic Immunity	II	Completed
DermaVir	NCT00270205	AIDS Clinical Trials Group	I/II	Completed [48]
DermaVir	NCT00712530	Genetic Immunity	I	Completed [49]
PENNVAX-B (Gag, Pol, Env)	NCT01082692	Inovio Pharmaceuticals	I	Completed [50]
PENNVAX-B ± IL-12 or IL-15	NCT00775424	University of Pennsylvania	I	Completed
MAG pDNA vaccine ± IL-12	NCT01266616	NIAID	I	Completed
DNA + lipopeptide vaccines				
GTU-Multi-HIV B Clade Vaccine + LIPO-5	NCT01492985	French National Institute for Health and Medical Research/French National Agency for Research on AIDS and Viral Hepatitis (Inserm/ANRS)	II	Active/closed to enrollment
DNA + viral vector vaccines				
MAG-pDNA + rVSVIN HIV-1 Gag	NCT01859325	NIAID/Profectus Biosciences, Inc.	I	Active/closed to enrollment
D-GPE DNA + M-GPE MVA	NCT01881581	Centers for Disease Control and Prevention, China	I	Unknown
JS7 DNA + MVA62B	NCT01378156	GeoVax, Inc.	I	Completed [HIV R4P 2014, Abstract OA05.03 (webcast)]
RNA-based vaccine				
iHIVARNA-01 (TriMix and HIV antigen naked messenger RNA)	NCT02413645	Biomedical Research Institute August Pi i Sunyer (IDIBAPS)	I	Active
Peptide-based vaccine				
VAC-3S	NCT02041247	InnaVirVax	II	Active/closed to enrollment
VAC-3S	NCT02390466	InnaVirVax	I/IIa	Active/closed to enrollment
Vacc-4x	NCT00659789	Bionor Immuno AS	II	Completed [51]
VAC-3S	NCT01549119	InnaVirVax	I/IIa	Completed (30 years of HIV Science, 2013, poster abstract 145; IAS 2015 toward an HIV Cure Symposium, Poster Abstract PE67 LB)
TUTI-16 (synthetic HIV-1 Tat epitope)	NCT01335191	Thymon, LLC	I/II	Completed [52]
Vacc-C5	NCT01627678	Bionor Immuno AS	I/II	Completed
AFO-18	NCT01141205	Statens Serum Institut (SSI)/ Ministry of the Interior and Health, Denmark/European and Developing Countries Clinical Trials Partnership	I	Completed [53]
AFO-18	NCT01009762	SSI/Rigshospitalet/Hvidovre University Hospital/Ministry of the Interior and Health, Denmark	I	Completed [54]
HIV-v	NCT01071031	PepTcell Limited	I	Completed [55]
(Lenti)-viral vector-based vaccine				
THV01	NCT02054286	Theravectys S.A.	I/II	Active/closed to enrollment

1.7.2 Dendritic cell vaccines

Dendritic cell vaccines have been explored over the last few years and represent a novel target for therapeutic vaccination. Dendritic cells (DCs) are potent antigen presenting cells (APCs) with a unique ability to induce both CD4+ and CD8+ responses [137]. Several mouse models have shown when autologous myeloid DCs, such as monocyte derived DCs (MD-DCs), are pulsed *ex vivo* with a variety of antigens, protective immunity can be induced including in an HIV-1 model [138].

To date over 10 clinical trials have been published using DCs in therapeutic vaccine for HIV-1 [139]. Perhaps the most encouraging results have been the use of high dose MD-DCs pulsed with heat inactivated autologous HIV-1 virus to assess whether HIV-specific immune responses could change plasma VL set point after treatment interruption. In this clinical trial 36 HIV-infected participants on ART were enrolled and received 3 immunisations with the regimen stated above or placebo. Results showed a highly statistically significant and consistent virological response and decrease in pVL set point in the vaccinated group for at least 24 weeks after treatment interruption. Using T cell ELISpots, consistent increases in HIV-1 specific T cell responses could also be measured [140]. Several other clinical trials using DC vaccines have also now been completed suggesting in HIV-1 infection DC immunotherapy can elicit HIV-1 specific immunological responses. However, it is important to note in this study VL rebounded to detectable levels in all patients.

1.7.3 Tat therapeutic HIV vaccine

Tat plays a key role in HIV virulence, replication, transmission and gene expression [141],[142]. Although it is produced early on in HIV infection, it continues to be expressed even in individuals on ART, exerting effects on the virus and the host immune system thus potentially making it an ideal candidate for therapeutic immunisation and ART intensification [143]. Importantly, anti-*Tat* Abs are infrequently found in natural infection but when present have been associated with high CD4+ counts, lower viral loads and prolonged asymptomatic states particularly when present in high titres [144]. All these findings suggest *Tat* could be used as a 'pathogen-driven' intervention to intensify ART.

In a recent Phase II trial, 168 HIV-infected participants on ART who were anti-*Tat* Ab negative were randomised to receive the HIV-1 *Tat* protein at 7.5µg or 30µg given either 3 or 5 times at monthly intervals [145]. Immunisation was found to be safe and well tolerated throughout the trial regardless of dose received. Immunogenicity results showed anti-*Tat* Abs were induced in 79% of vaccinees, and was higher in the 30µg group (92%) in particular after 3 immunisations. With respect to immune restoration measured by an increase in CD4+ cells, there was a mean increase of CD4+ cells observed

in vaccinees out to 3 years of follow up, as well as increases in numbers of B cells, NK cells and improvements in CD4+/CD8+ ratios. In addition, *Tat* immunisation induced a statistically significant reduction in HIV DNA that also persisted out to year 3.

An important limitation of this study was it was not a placebo controlled double blinded trial. However a parallel study was conducted examining standard of care (triple therapy) without *Tat* vaccination in matched individuals, therefore the authors maintain these preliminary results indicate *Tat* could play a vital role in restoring immune balance in HIV infection and intensifying ART.

1.7.4 Therapeutic HIV antibodies

Moving away from conventional vaccination methods, passive immunization of monoclonal antibodies have shown some early signal in HIV clinical trials. Immunotherapy is not a new modality to treat infectious disease, and monoclonal antibodies are currently being used to treat other disease modalities such as cancers and autoimmune diseases, through mechanisms engaging the host Fc receptors [146]. In view of efficacy results from pre-clinical trials in humanized mice and macaques, it has been suggested that broadly neutralizing antibodies (bNAbs) could be used in HIV infected individuals [147],[55].

In a Phase I open label clinical trial a potent anti-CD4+ binding site antibody (3BNC117) was cloned from a viraemic controller and administered intravenously (IV) at different doses ranging from 1mg/kg to 30 mg/kg to 12 uninfected and 17 HIV-infected individuals [148]. Results showed the Ab to be safe and well tolerated with no dose-dependent increase in adverse events. Although CD4+ and CD8+ cell counts did not change after infusion in the HIV-infected volunteers, there were highly significant and rapid decreases in viral loads in those receiving the highest doses (-0.8 to -2.5 $\log^{10}$). The data did not allow viral set point determination in this cohort, but the authors concluded the drop in viraemia from baseline was highly significant until day 28, and that passive infusion of single bNabs could have profound effects on HIV viraemia, a potential alternative to the elusive therapeutic vaccine.

A similar monoclonal Ab VRC01 which also targets the conserved CD4+ receptor binding site on gp120 and is thought to neutralize approximately 90% of diverse virus strains [149] has also completed Phase I. In this trial infected individuals both on (15 subjects) and off ART (8 subjects) received 1 or 2 infusions of VRC01 IV or subcutaneously (SC) [150]. Safety data was again reassuring with no dose related toxicities.

In HIV-infected individuals on ART, infusion with VRC01 did not affect levels of total and integrated HIV DNA. However, in the participants not on ART a single dose of VRC01 was shown to decrease

plasma VL in 6/8 of the subjects, with 2 subjects who had the lowest baseline VLs maintaining undetectable plasma VLs post-infusion for over 20 days. This data again provides proof of concept for the effect of monoclonal antibodies on achieving viraemic control.

1.8 HIV CURE

1.8.1 Sterilising cure

Since the publication of the only 'cured' patient to date in 2009, there have been no further cases of a complete sterilising cure. Timothy Ray Brown also known as the 'Berlin' patient was diagnosed with HIV in 1995, and subsequently developed acute myeloid leukaemia (AML) 10 years later [151]. After recurrence of the AML following chemotherapy, the only chance of survival was to receive a stem cell transplant. At the time the medical team found a donor match who was also homozygous for the CCR5 delta32 deletion, which has been shown to confer high (although not complete) resistance against HIV acquisition [152], estimated to be present in only 1% of the Caucasian population.

At the time of transplantation all ART was stopped, with the patient's PBMCs showing a change to the new homozygous CCR5 delta32 allele. Furthermore, HIV-1 could not be detected in peripheral blood nor mucosal tissue assessed with RNA and proviral DNA PCR assays [153]. To date Timothy Ray Brown remains off ART, HIV free and 'cured.'

Following the unexpected success of this, 2 chronically infected HIV patients in Boston with lymphoma underwent stem cell transplantation. Both of these patients were heterozygous for the CCR5 delta32 deletion, but received HIV-1 susceptible wild type donor cells (different to the above case of Timothy Brown). Although post-transplant substantial reductions in HIV reservoir were observed in these two patients whilst they were on ART, after a brief period of treatment interruption both patient's viral loads rebounded and they were restarted on treatment [154].

1.8.2 Functional cure

Post-treatment control or 'functional cure' where the virus is controlled after ART is stopped remains an exciting and perhaps more realistic goal for HIV cure. In the VISCONTI study 14 HIV positive patients were started on ART very early within 10 weeks of primary HIV infection (PHI), and achieved undetectable viral loads within a median of 3 months. ART was then stopped after a median of 36 months of treatment, and this cohort was found to control virus after the

discontinuation of ART for several years [155]. Control of viraemia in these post-treatment controllers (PTCs) was associated with very low level HIV reservoirs, with the authors speculating early ART initiation and a lengthy treatment period playing an important role in this.

Further excitement surrounding functional cure grew after a case report where an infant born to an HIV positive women who had received no antenatal care and was therefore not on treatment for HIV, was treated with triple ART (zidovidine, lamivudine and nevirapine) within 30 hours of birth [156]. The infant went on to achieve an undetectable viral load where she was then lost to follow up and reported to have stopped ART at 18 months. On return to care, the infant was shown to have been able to control HIV viraemia whilst off therapy for the last 12 months, with no evidence of HIV RNA or circulating HIV antibodies. This excitement however has now turned to disappointment and although the infant was able to maintain control of viraemia for several more months, she is now back on treatment following viral rebound.

A similar case study in Milan has now also shown viral rebound despite early ART therapy in the infant (within 12 hours), the absence of HIV Ab, DNA, p24, and HIV-RNA at 3 years. Here ART was stopped but viral load rebounded within 2 weeks [157].

Recent data from macaque studies have indicated perhaps a functional cure will be harder to achieve than previously thought due very early seeding of the reservoir. In this study 20 macaques were infected rectally with HIV and triple therapy was initiated on days 3, 7, 10 and 14 following infection (with tenofovir, emtricitabine and dolutegravir), with a control group receiving no ART [158]. Macaques receiving ART treatment at day 3 showed no detectable viraemia at any time point over the 24 week treatment period (4/4 monkeys). Furthermore, these macaques had no evidence of proviral DNA in PBMCs at any time point in comparison to those started on later treatment.

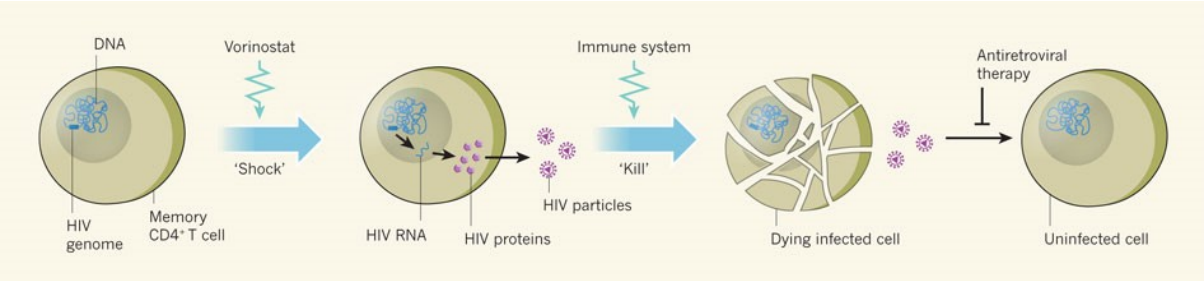
At week 24 ART was discontinued in all macaques. Although there was a three-fold delay in viral rebound in the animals started on very early ART, all macaques displayed plasma viral load rebound. Although this animal data cannot be directly extrapolated to human viral reservoir kinetics, it has provided important data to suggest the viral reservoir can seed substantially earlier than previously thought and even before detectable viraemia, which may have important implications for future eradication strategies.

1.8.3 Combination approaches to HIV cure- role of the therapeutic vaccine

Perhaps the macaque data provides good evidence for the inability of early ART alone to control virus and result in a functional cure. Newer combination approaches include using histone deacetylase inhibitors (HDACis) to attempt to reduce or eliminate the viral reservoir, with early data

showing the ability of some potent HDACis such as vorinostat able to disrupt HIV latency [159]. However, this alone may also not be enough to eradicate infection, and it is here where a therapeutic vaccine could play a key role in the combination approach. The 'kick and kill' method describes the use of an anti-latency agent to activate the viral reservoir, in combination with a vaccine to enhance or prime the immune response to clear replicating virus (Figure 1.9) [160]. This eradication strategy is currently under investigation using a vorinostat/vaccination combination in patients started on ART in primary infection (MRC RIVER trial).

Figure 1.9. Diagram to represent the 'kick and kill' approach using voronistat. Adapted from Deeks. HIV: Shock and kill. Nature 2012; 487: 439-440.



1.9 DNA VACCINATION IN HIV PREVENTION AND THERAPEUTICS

DNA vaccines are the focus of my MD(Res) project, and have the potential to induce cytotoxic T lymphocytes (CTLs), stimulate T helper cells and induce antibody responses providing an attractive vaccine option [161]. In pre-clinical studies they have been shown to elicit strong and often protective immune responses against a host of pathogens including viruses, bacteria, autoimmune disease and cancer, and various DNA vaccines now been licensed for use in animals [162].

To induce an immune response, the gene sequence encoding the vaccine is inserted into the plasmid and expressed in the host cell. The majority of the DNA is degraded, but a small amount enters cells at the injection site and is expressed by local APCs [163]. Once inside the cell, the DNA travels to the cell nucleus, and depending on the DNA uptake, route of delivery and whether adjuvants are present to enhance uptake into cells (discussed below), the adaptive immune response to the encoded gene is triggered [164].

The main advantages of using DNA include cost effectiveness and ease of production [165], heat stability which could be crucial in developing countries where cold storage is not always available [166], and well documented safety in both preclinical and human trials with low incidence of DNA integration [167].

Despite these clear advantages few studies have shown efficacy in human trials, and one major disadvantage is the uptake of DNA by cells *in vivo* is inefficient, with most of the intramuscularly injected DNA via conventional methods of vaccination not actually able to transfect cells [168]. This major limitation has focused much attention on improving DNA uptake into cells through a number of different methods and delivery routes.

1.9.1 Optimising uptake of DNA

Prime boost combinations

Limitations in the use of DNA as individual agents in clinical trials has led to growing investigation into work using DNA as a priming immunization, usually combined with recombinant attenuated viruses expressing the vaccine antigen, such as adenovirus as previously mentioned and modified vaccinia Ankara (MVA). Data from the HVTN 068 HIV vaccine trial evaluated the immunogenicity of different prime-boost strategies using DNA and rAd5 compared with a regime excluding DNA [169]. Although in this particular trial DNA did not augment CD4+ or CD8+ responses, the potential for DNA as a primer is currently being widely investigated.

Chemical methods to improve uptake and immunogenicity of DNA

Liposomes translate to 'small lipid vesicles' which can aid DNA delivery *in vivo*, with several pre-clinical studies contributing to more effective transfection than with naked DNA [170]. Their main mechanism of action is to interaction with the cell membrane, preventing extracellular degradation and therefore can increase DNA transfection into DCs. Similarly as the name suggests virosomes can be used to encapsulate DNA but also contain viral envelope proteins to allow for fusion with target cells followed by DNA delivery into the cell [171]. In macaque models these virosomes have shown some protective mucosal IgA responses against HIV infection, but this is yet to translate to clinical trials [172].

Other promising chemical methods of improving DNA delivery include the newer nanoparticles. These are biodegradable polymers which compact DNA into nanoparticles and are then used to efficiently deliver DNA into cells [171]. Pre-clinical trials using nanoparticles are currently in progress and are thought to have great potential in future DNA vaccine strategies.

Physical methods

Several physical methods have been examined to increase the efficacy of DNA delivery into cells, and although these methods have not been compared to each other in trials, they have been compared in single studies to conventional IM methods of DNA vaccination.

The Biojector device for example, utilises a pressured gas (usually carbon dioxide) to penetrate the skin in the absence of a needle. In both preclinical (malaria and dengue trials) [173], [174] and clinical trials (Hepatitis A) [175] this device has been used to deliver DNA and induce greater T and B cell responses that IM delivery. In the context of HIV, a recent Phase I trial using the Biojector device for DNA priming showed the device made significant contributions in improving immunogenicity, supporting continued clinical evaluation of this device for future DNA vaccine studies [176].

The gene gun delivery system similarly uses a high pressure gas to deliver DNA-coated gold particles usually via the ID route. These gold particles penetrate cell membranes resulting in increased uptake by cells and when performed ID has been show to allow greater transfection of APCs [177]. Other examples of physical methods of DNA delivery are microneedle arrays which use patches of tiny needles to penetrate the skin. These needles can be pre-coated with DNA or DNA can be delivered through hollow microneedles after skin penetration [178],[179].

Arguably the most significant improvement in delivery of DNA vaccines has been the use of electroporation (EP) [180]. EP is the application of electrical pulses to create an electric field at the site

of injection causing a temporary cell membrane instability to allow DNA (and other molecules) to enter cells and tissues. Although the exact mechanism by which EP works is not fully understood, it is thought the inflammation associated with the procedure aids APC recruitment to the vaccine site and can influence antigen presentation [181].

The first randomised clinical trial to demonstrate the importance of EP was placebo-controlled Phase I clinical trial of the ADVAX Clade C/B DNA vaccine in HIV uninfected volunteers where IM vaccine administration was compared to IM+EP administration at 3 different doses to assess whether EP could enhance immunogenicity compared to standard IM injection [182]. 40 volunteers were enrolled into this study, receiving either high dose IM vaccination (4mg), placebo, or differing doses of vaccination delivered by EP (range 0.2mg-4mg) 3 times in total over a 6 month period. Overall ADVAX delivered by standard IM injection or by EP was safe and well-tolerated. Importantly both 'intense' and 'severe' pain were reported by volunteers immediately following EP, but 97% of volunteers regarded EP as an acceptable form of vaccine delivery for a serious infection such as HIV in tolerability questionnaires.

Immunogenicity data from T cell ELISpot assays showed EP significantly improved the cellular immune response rate in terms of magnitude, duration and breadth of response to multiple antigens encoded by the vaccine (88% volunteers in the 4mg EP group to at least 1 antigen, compared with 0% in the 4mg IM group and 0% in the placebo group). The highest magnitude was an anti-*Env* response in the high dose EP group compared to the same dose received by IM injection (70-fold higher response in the EP group). Phenotyping of the responses by ICS demonstrated this was a predominantly CD4+ T cell response. There were limited antibody responses seen in this trial which the authors concluded were likely a reflection of the ADVAX vaccine (primarily a T cell vaccine) rather than the effectiveness of the EP procedure.

More recently, intradermal EP used in combination with HIV-DNA has been investigated using an integrated ID device. In this trial of HIV uninfected volunteers, 25 volunteers received 3 immunisations using the Zetajet ID device with or without EP, and immunogenicity was compared between groups and with a placebo group [183]. The regimen consisted of an HIV-DNA prime (encoding Env gp160 of HIV subtypes A, B and C, Gag A and B and reverse transcriptase of subtype B), HIV-MVA boost, with a further subgroup receiving CN54 subtype C gp140 protein at the time of MVA vaccination.

Results showed pain scores to be lower than with IM+EP and in general all vaccinations were well tolerated. After 3 DNA immunisations IFN-ɣ ELISpot responses to *Gag* were found to be only modest,

with no statistical differences reported between groups that received ID vaccination alone compared to ID+EP.

To date, EP combined with DNA has only been used in one clinical trial in HIV-infected volunteers. In the AIDS Clinical Trials Group (ACTG) A5281 the safety, tolerability and immunogenicity of a multiantigen (MAG) DNA vaccine combined with increasing doses of IL-12 plasmid and delivered by EP was investigated in healthy HIV-1 infected adults (nadir CD4>200 cells/mm^3) who had undetectable VLs on ART [184]. Four groups of participants were randomly allocated to receive either MAG-DNA vaccine (or placebo) IM with EP, with progressively higher doses of IL-12 according to group (range of IL-12 0-1000µg).

Overall in this HIV infected cohort, HIV MAG-DNA vaccine enhanced with IL-12 and delivered IM+EP was safe and well tolerated. There were modest but significant increases in CD4+ T cell response as measured by ICS against multiple HIV antigens in the group receiving the lowest dose of IL-12 in combination with MAG-DNA delivered IM+EP. No changes were seen across any group with any dose of IL-12 in the CD8+ T cell populations examined. The authors concluded the results of this study were modest although consistent with a similar study in an HIV uninfected cohort [185].

1.9.2 The importance of the route of DNA delivery- targeting the skin

The route of DNA delivery can play key role in increasing efficacy and improving immunogenicity. There are two main tissue targets for parenteral vaccination: the skin (consisting of the epidermis and dermis) and the muscle. The skin is densely packed with important APCs including Langerhan's cells (LCs) and DCs which have the ability to migrate to draining lymph nodes and trigger immune responses [186]. Although IM vaccination remains the most popular route of vaccine delivery in clinical trials, intradermal and transcutaneous vaccination techniques are gaining popularity and are of particular interest and relevance to this research project.

Intradermal (ID) delivery

Intradermal vaccine delivery has been extensively studied in trials based on protein vaccines rather than DNA. A higher density of intradermal APCs and their efficient loading is a major advantage of vaccination into the intradermal layer, and thought to be crucial for T cell priming [187]. Both animal and clinical trial data have also suggested ID vaccination can be dose-sparing with some vaccines only requiring one-fifth of equivalent dosing to be as immunogenic [188], [189]. A few studies have compared intradermal (ID) to intramuscular (IM) routes of vaccination and have suggested IM delivery may have limitations compared with ID due to inability of myocytes to adequately take up

DNA before breakdown in the peripheral circulation [190]. However, few trials have compared ID in combination with IM DNA vaccination, with or without EP.

Transcutaneous vaccine delivery

Transcutaneous vaccination (TC) refers to the topical application of antigens onto the epidermis and is aimed at targeting Langerhan's cells abundant in the epidermis, rather than DCs [186]. These LCs have been shown to be potent in inducing CD8+ cells in *in* vitro studies [191]. TC techniques are minimally invasive and do not require penetration of the skin with needles, providing an attract vaccine delivery method. Despite these advantages, the use of TC vaccination has been underexplored for other modes of vaccine delivery in clinical trials, and as yet not been utilised with DNA vaccination.

A major challenge with TC immunisation is although the skin layer is rich in LCs, these are in close proximity to the tough outer stratum corneum skin layer [192]. To disrupt this skin layer and optimise antigen presentation, several techniques have been developed including thermal abrasion, shaving, friction, the use of sand paper, and tape stripping [186]. More recently a technique known as cyanoacrylate skin surface stripping (CSSS) using a combination of shaving, glue, and adhesive tape has been shown to remove approximately 33% of the stratum corneum by unplugging hair follicles and removing debris, and has been shown to be well tolerated in early human clinical protocols [193].

In a previous clinical trial using TC immunisation and CSSS with an inactivated influenza vaccine (Tetragrip), TC vaccination was compared to IM injection, first in 24 HIV uninfected volunteers, and subsequently in 12 HIV infected volunteers [191]. In the first cohort, ICS showed the transcutaneous route of immunization induced both CD4+ and CD8+ responses with a preferential induction towards CD8+ responses compared to IM. IM vaccination as expected demonstrated strong humoral immune responses (by measurement of NAbs) although there were no NAb responses in those receiving TC immunisation. Similar findings were present in the HIV positive cohort, demonstrating the potential importance of inducing anti-viral CD8+ responses in infections such as HIV, with a potential dose-sparing effect. These findings have also been shown using CSSS and TC immunisation with another influenza vaccine (Aggripal) in 11 healthy volunteers, with similar findings [194].

1.10 THESIS HYPOTHESIS AND AIMS

My MD(Res) project is based almost entirely on the recruitment and retention of volunteers to both a preventative (CUTHIVAC 001) and a therapeutic (CUTHIVTHER 001) vaccine trial using a GTU®MultiHIV B clade DNA vaccine. This investigational GTU®MultiHIV B clade DNA vaccine is a synthetic fusion protein comprising of full-length polypeptides of *Rev, Nef, Tat, p17/p24* and *CTL* (containing epitopes of protease, reverse transcriptase and gp160) regions of the HAN-2 HIV B clade virus [195]. The vector developed by FITBiotech is a non-replicating expression vector with enhanced features provided by the bovine papilloma virus transcriptional activators and segregation/partitioning factor E2 protein along with its multimeric specific sites [196].

The vaccine is designed to induce immune responses to HIV-1 regulatory genes *Rev, Nef, Tat, Gag* and *CTL* epitopes coded for by *pol* and *env* genes. As previously stated, this DNA vaccine demonstrated encouraging results in the FIT-06 Phase II trial in South Africa, where a cohort of HIV-1 Clade C infected participants who were healthy and not yet on ART were enrolled. Participants were allocated to either vaccine or placebo groups with IM or ID administration of the vaccine. Significant declines in viral load (0.5 log decrease) and increases in CD4 counts were more pronounced in the IM group and in particular HLA genotypes (predominantly B*5703), with significant increases in HIV specific CD4+ and CD8+ cells post vaccination in both groups [135].

Hypothesis

1. DNA vaccination induces HIV-specific T cell immunity in both HIV negative and HIV positive volunteers, and is influenced by the route of vaccine administration
2. DNA vaccination induces HIV-specific immunity that confers protection in a viral challenge *in vitro* system in HIV negative and HIV positive volunteers

Aims/Objectives

1. To characterise T cell responses in HIV negative and HIV infected volunteers immunised with a DNA vaccine, and to determine whether T cell responses are influenced by the route of vaccine administration
2. To determine functionality of HIV-specific T-cell responses in a novel *in vitro* viral inhibition assay amongst HIV negative and HIV infected volunteers immunised with an HIV DNA vaccine

Chapter 2: Materials and Methods

2.1 CUTHIVAC 001: STUDY SUBJECTS AND VACCINE SPECIFICS

CUTHIVAC 001 was a phase I randomised controlled clinical trial aimed at investigating the safety and immunogenicity of three GTU®MultiHIV B clade DNA vaccine immunisations administered intramuscularly (IM), and enhanced by intradermal delivery (ID), transcutaneous delivery (TC) and electroporation (EP).

2.1.1 Study Participants

All study visits were conducted at the Clinical Trials Centre, St Mary's Hospital London. Healthy adults aged between 18-45 years determined to be at low risk for HIV-1 infection and with no clinically significant medical history were enrolled into the study (Table 2.1).

2.1.2 Ethical approval

The CUTHIVAC 001 trial was conducted in compliance with UK Clinical Trial Regulations and within the principles of Good Clinical Practice (GCP). The study was approved by the National Research Ethics Service, Cambridge East Research Ethics Committee and by the Medicines and Healthcare products Regulatory Authority (MHRA). All participants provided informed written consent after thorough counselling by me as co-investigator on this trial.

2.1.3 Vaccine candidate and administration

The investigational GTU®MultiHIV B clade DNA vaccine is a synthetic fusion protein comprising of full-length polypeptides of *Rev, Nef, Tat, p17/p24* and *CTL* (containing epitopes of protease, reverse transcriptase and gp160) regions of the HAN-2 HIV B clade virus [195]. This vector developed by FITBiotech is a non-replicating expression vector with enhanced features provided by the bovine papilloma virus transcriptional activators and segregation/partitioning factor E2 protein along with its multimeric specific sites [196].

Participants were randomised into one of 3 groups and vaccinated at weeks 0, 4 and 12 (Table 2.2). Group 1 received ID+IM vaccination, group 2 TC+IM vaccination, and group 3 received IM vaccination with EP delivered immediately after vaccination at the same site. All vaccine visits and administrations were conducted by me as the co-investigator and/or a research nurse.

Table 2.1: Inclusion/exclusion criteria CUTHIVAC 001

INCLUSION CRITERIA	EXCLUSION CRITERIA
Men and women aged between 18-45	Pregnant or lactating
BMI between 19-30	Use of topical treatment on injection or application site within last 4 weeks
Willing and able to given informed written consent	No UV tanning sessions or strong sun exposure within 4 weeks prior to study
At low risk of HIV and willing to remain so for the duration of study defined as: • No history injecting drug use in last 10 years • No gonorrhoea or syphilis infection in last 6 months • No 'high risk' partner (eg IDU, HIV positive) currently or in last 6 months • No UPAI in last 6 months outside of relationship with regular HIV negative partner • No UPVI in last 6 months outside of relationship with regular HIV negative partner	Clinically relevant abnormality on history or examination including: • History of seizure disorder • Severe eczema • Liver disease with inadequate hepatic function • Any skin condition which may interfere with injection site • Haematological, metabolic, gastrointestinal, cardiac disorders including abnormal ECG • Autoimmune disease or use of immunosuppressives in last 3 months
If heterosexually active female using an effective method of contraception (includes consistent condom use), if heterosexually active male using effective method with partner	Excessive terminal hair growth on investigational areas
	Grade 1 or above abnormal laboratory parameters (including conjugated hyperbilirubinaemia)
Agree to abstain from blood donation for 3 months after the end of trial or longer if necessary	Skin fold measurement on upper thighs >40mm
	HIV-1 or 2 positive, HepBsAg positive, HepC Ab positive
	Previous severe reaction to vaccination or receipt of live attenuation vaccine in last 60 days

Key: BMI=body mass index; UV=ultraviolet; IDU=injecting drug use; UPAI=unprotected anal intercourse; UPVI=unprotected vaginal intercourse; ECG=echocardiogram; HepBsAg=hepatitis B virus surface antigen; HepC=hepatitis C virus

Table 2.2: Vaccine schedule for CUTHIVAC 001 including groups, route of vaccination and cumulative doses of DNA.

Group	Route and Vaccine Schedule			Cumulative dose DNA
	Week 0	Week 4	Week 12	
1. ID + IM (n=10)	0.4mg ID 4mg IM	0.4mg ID 4mg IM	0.4mg ID 4mg IM	13.2mg
2. TC + IM (n=10)	0.4mg TC 4mg IM	0.4mg TC 4mg IM	0.4mg TC 4mg IM	13.2mg
3. EP + IM (n=10)	4mg IM	4mg IM	4mg IM	12mg

Key: ID=intradermal; IM=intramuscular; TC=transcutaneous; EP=electroporation.

Intradermal Vaccination (Group 1: ID+IM)

In group 1 (ID+IM) participants received 2mg (1ml) IM injections of GTU®MultiHIV B DNA into each upper thigh to total 4mg (2mls) at weeks 0, 4 and 12. In addition, participants in this group also received a further 0.2mg (0.1ml) vaccine delivered intradermally into each upper arm to total 0.4mg (0.2mls). A small needle (25 or 27 gauge) was inserted into the dermis of each upper arm below the deltoid muscle. The 'bleb' or weal created was used as a marker of accuracy and to ensure consistency of injection into the intradermal layer.

Transcutaneous Vaccination (Group 2: TC+IM)

In group 2 (TC+IM) participants received 2mg (1ml) IM injections of GTU®MultiHIV B DNA into each upper thigh to total 4mg (2mls) at weeks 0, 4 and 12. In addition, a novel needle-free transcutaneous method was used to deliver a further 0.4mg (0.2mls) of the vaccine onto one upper arm below the deltoid muscle. To overcome the stratum corneum, the skin layer was disrupted using a process known as cyanoacrylate skin surface stripping (CSSS) where superglue was placed onto a predefined area on the upper arm (5 x 3.2cm), and adhesive tape place over the area. The glue was then left to dry for 20 minutes and stripped off. This technique opens the hair follicles and removes debris and sebum [193]. The vaccine was then dripped on to the pre-defined site, massaged in using a finger cot for 1 minute, and allowed to absorb into the skin for 20 minutes. A clear bandage was placed over the arm and participants were advised not to wash for 24 hours and to avoid excessive sweating until the bandage was removed at the safety visit the following day.

Intramuscular Vaccination with Electroporation (Group 3: EP+IM)

In group 3 (EP+IM) participants received 2mg (1ml) IM injections of GTU®MultiHIV B DNA into each upper thigh to total 4mg (2mls) at weeks 0, 4 and 12. In addition and directly following IM injection, participants also received electroporation (EP) at the same site using the TriGrid integrated device (Figure 2.1). The disposable EP cartridge was loaded with GTU®MultiHIV B DNA vaccine then adjusted to one of three depth settings, corresponding to pre-defined ranges in skin fold thickness. The cartridge was then loaded into the handheld EP device and applied to the vastus lateralis muscle of each upper thigh. IM administration of GTU®MultiHIV B was followed immediately by the application of electrical stimulation. The spacing of the TriGrid electrode array was 6 mm in a diamond-shaped configuration, and the electrical field was applied at an amplitude of 250 V/cm over a 40 millisecond total duration, resulting in brief muscle contractions. All electroporation procedures were performed by me or one of the trained research nurses.

Figure 2.1: A: Integrated hand held device used to both deliver IM injection plus electroporation immediately following injection. B: Diagram showing injection needled surrounded by electrodes in a diamond formation through which electroporation is delivered. Images adapted from www.ichorms.com

2.1.4 Reactogenicity and safety

Participants returned for a 24 hour and 7 day safety visit, where they were monitored for local and systemic reactogenicity and adverse events. In addition, participants filled out diary cards from 12 hours to 7 days post vaccination. Those randomised to EP+IM were asked to fill out a pain questionnaire rating their experience of the procedure, using a visual analog pain score (0=no pain, 10=worst pain ever), and also conducted an acceptability questionnaire relating to the procedure.

2.1.5 Immunogenicity sampling schedule and PBMC processing

Blood samples were collected from participants at specified time points (Table 2.3), and peripheral blood mononuclear cell (PBMC) processing was conducted according to the standard operating procedure (SOP) for blood processing, PBMC, serum and plasma collection to Good clinical laboratory practice standards (GcLP). Samples were processed for serum and PBMC fractionation and stored in batches at either-20 or -80˚C respectively until I analysed them for immunogenicity outputs.

Table 2.3: Immunogenicity sampling schedule for serum, PBMCs and mucosal samples: CUTHIVAC 001

Visit number	1	2	3	4	5	6	7	8	9	10	11
Nature of visit	Screen	Dosing visit	Safety visit	Safety visit	Dosing visit	Safety visit	Safety visit	Dosing visit	Safety visit	Safety visit	Follow-up visit
Week	-6	0	0+1day	1	4	4+1day	5	12	12+1day	14	20
Blood for serum – immunogenicity	X	X			X		X	X		X	X
Mucosal sampling - immunogenicity		X								X	
Blood for PBMC – immunogenicity		X		X	X		X	X		X	X

2.2 CUTHIVAC 001 IMMUNOGENICITY: T CELL IFN-ɣ ELISPOT ASSAY

2.2.1 Reagents and buffers

R20: used for thawing PBMCs and subsequent rest overnight at 37˚C/5% CO_2. R20 was prepared by addition of the following reagents:

1. RPMI 1640 medium (Sigma) bottle with 110mls removed
2. 100ml thawed heat-inactivated foetal calf serum added (HI-FCS, batch controlled)
3. 5mls of 100 x penicillin-streptomycin solution (Gibco): 100units/ml penicillin, 100µg/ml Strep
4. 5mls of L-glutamine (Gibco): 2mMol

The R20 was filtered (0.22 µm filter used) under sterile technique and stored at 2-8°C.

R10: used as Assay Medium, and prepared by addition of the following reagents:

1. RPMI 1640 medium (Sigma) bottle with 60mls removed
2. 50ml thawed heat-inactivated foetal calf serum added (HI-FCS, batch controlled)
3. 5ml of 100 x penicillin-streptomycin solution (Gibco): 100units/ml penicillin, 100µg/ml Strep
4. 5ml of L-glutamine: 2mMol

The R10 was filtered (0.22 µm filter used) under sterile technique and stored at 2-8°C.

Peptides

15-mer overlapping peptides (11 amino acid overlap) were supplied by FITBiotech and custom synthesized by ProImmune, Oxford, UK (Appendix 1).

Peptide pools: (*Rev, Tat, Nef, Gag, CTL*). A stock concentration of 1mg/mL was initially prepared, with the final concentration following dilution of 5µg/mL (2.5µg/mL) in the well.

Positive controls

Phytohaemagglutinin (PHA). A stock concentration of 50µg/mL was diluted in R10 to give the final concentration required of 5µg/mL (2.5µg/mL) in the well.

Cytomegalovirus/Epstein-Barr virus/influenza/tetanus T cell epitopes (CEFT). A stock concentration of 25µg/mL was diluted in R10 to give the final concentration required of 5µg/mL (2.5µg/mL) in the well.

IFN-ɣ ELISpot kits

All T cell ELISpot kits were ordered in bulk from Mabtech from the same batch.

IFN-ɣ T cell ELISpot kits: Mabtech 3420-4APT-4, Lot number: 286, Expiry: 08/2017

Streptavidin ALP and Mouse Ab: Mabtech 3420-4APT-4, Lot number: 286, Expiry: 08/2017

BCIP/NBT substrate: Mabtech 3650-2, Lot number: 286, Expiry: 08/2017

2.2.2 IFN-ɣ ELISpot Assay

Optimisation

The T cell ELISpot assay was previously optimised, standardised and qualified in the GcLP lab prior to my MD(Res) project, with a resulting SOP for application to human clinical trial samples written. In brief: a series of single donor NC24 leucocyte blood cones (N=17) were obtained via the NHS blood and Transfusion Services from uninfected volunteers, fractionated to obtain PBMC samples and frozen in batches in vapour phase nitrogen (-80°C) until use. These PBMC samples were used to optimise the ELISpot assay steps and to set background or 'non-response' levels as well as set positive control parameters. The IFN-ɣ ELISpot assay uses reagents supplied by Mabtech™ in addition to optimised peptide pools encoded by the vaccine and positive and negative controls as described above.

Following assay qualification, the T cell ELISpot assay was conducted as previously described [197],[198] and is shown below in diagrammatic form (Figure 2.2). Full details of the SOP are included in the appendix (Appendix 2). I conducted all steps and all ELISpot assays independently.

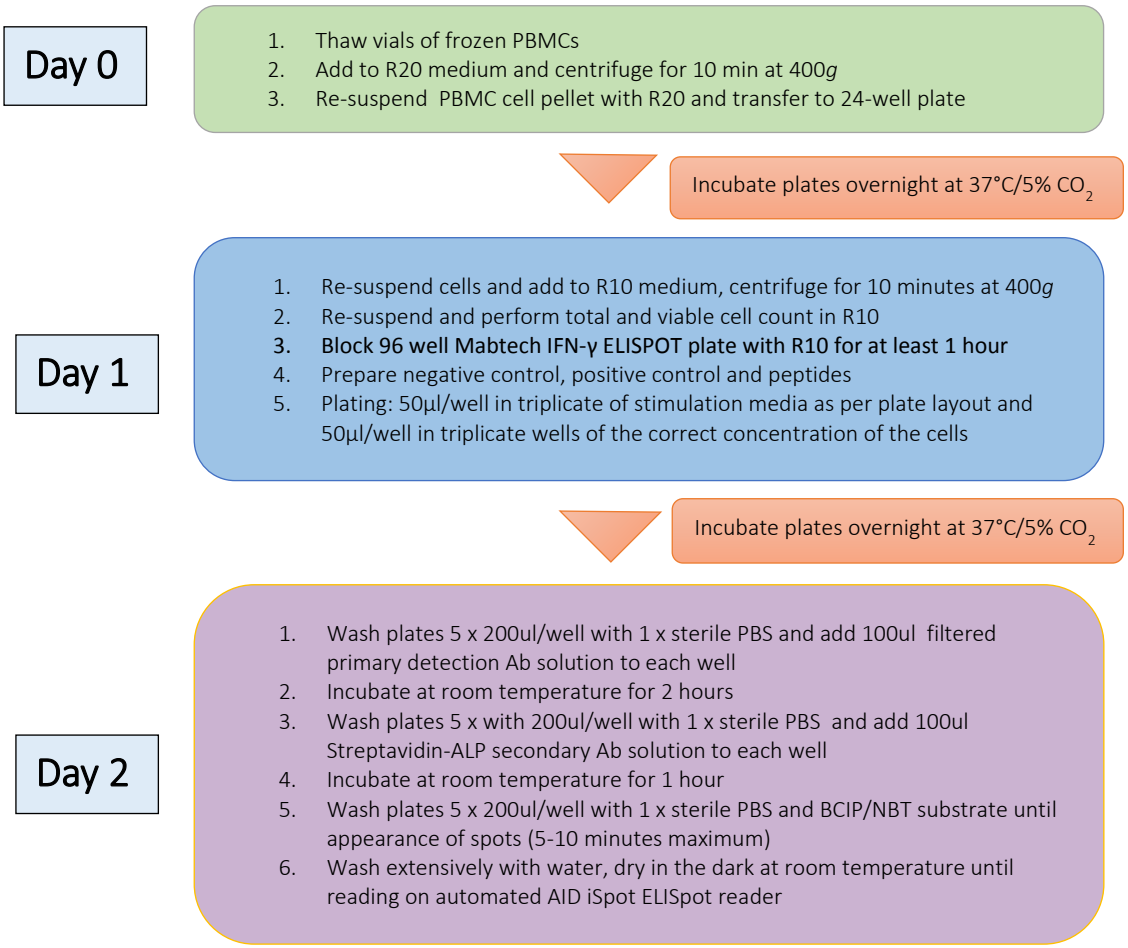


Figure 2.2: Schematic of T cell ELISpot assay

Pass/Fail Criteria

For the positive controls:

PHA was included as a positive control. If there were less than 10 spots per well for PHA, an assay reagent problem was likely and all samples on the assay plate in question retested. CEFT was also included as a peptide pool positive control. However, this may produce a variable response in participants, but again if there were less than 10 spots per well for CEFT an assay reagent problem suspected and samples retested.

For the negative control:

The negative control wells consisted of cells plus R10 alone with no stimulation. If there were more than 20 spots per well, these samples were retested.

Definition of a 'positive' response

All samples were conducted in triplicate wells. The mean number of spot forming units per million PBMC (SFU/M) was calculated by multiplying the mean number of spots in the triplicate wells by 5 (x50 for the PHA wells due to the 1:10 dilution factor). The final IFN-ɣ value was calculated as the mean count minus the background count (that is; the count present in the corresponding negative control well) and expressed as spot-forming units per million cells (SFU/M).

A response was considered positive if the following criteria were met: 1) The positive control was >1000 SFU/M; 2) The mean of the negative wells was <100 SFU/M; 3) The positive response was >4 x mean background (or greater than 0 if the background was 0) and >55 background subtracted SFU/M. These definitions have been applied to previous HIV-1 vaccine trials [199],[200].

2.2.3 Statistical Analysis

All statistical analysis was conducted using *Graphpad Prism* version 6.0.

For the immunogenicity analysis, the primary end point was defined as 2 weeks after the third and final vaccine (week 14).

The difference in magnitude of the INF-ɣ response between the groups at the specified time points including the primary end point for each peptide was compared using an unpaired t test (non-

parametric Mann-Witney test). A result was considered statistically significant if the 2 tailed P value was <0.05.

To analyse the difference within each individual group between week 0 and week 14, a paired t test (Wilcoxon matched-pairs singed rank test) was used with statistical significant set at p=<0.05.

2.2.4 Supporting work: Intracellular cytokine staining (ICS)

T cell responses were further evaluated by multi-parametric ICS with two 11 colour panels, one for CD8+ and one for CD4+ according to standard protocols.

The ICS assays were conducted by Celine Yan and Suzanne Venables (Research Technicians, Shattock group, Department of Medicine Imperial College London). Full details of the methods are shown in the SOP included in the appendix (Appendix 3).

The ICS data is presented as a percentage total of CD4+ or CD8+ cells with all background responses from negative controls subtracted. For the CD4+ results data for cytokines IFN-ɣ, IL-2, TNF-α and CD154 is presented, and for CD8+ cytokines IFN-ɣ, IL-2, TNF-α, CD57, granzyme B and perforin.

Although I did not conduct the ICS, a correlation between this data and my data from the T cell ELISpots is discussed in the results chapter as part of supporting work. I conducted the correlation analysis and it is important to include this data when interpreting the T cell immunogenicity results. The correlation analysis was conducted using *Graphpad Prism* version 6.0.

2.3 CUTHIVAC 001: VIRAL INHIBITION ASSAY

2.3.1 Reagents and buffers

For assay optimisation

Reagent	Vendor-Catalogue Number	Storage temp
Benzonase Nuclease	Sigma- E1014	-20°C
Trypan Blue	Sigma-T8154	Room temperature
1 x PBS	Gibco-14190	Room temperature
Human Serum	Sigma-H4522	-20°C
Comp Beads Anti-mouse Ig, κ	BD-552843	2-8°C
Formaldehyde	Polysciences, Inc.-04018	Room temperature
HI-FBS	Gibco-10108-165	-20°C

Bispecific antibodies

The bispecific antibodies used in the VIA optimisation process were obtained from the National Institute of Health (NIH):

122777 Anti-Human CD3/8 Bispecific Monoclonal Ab lot no: 140083- National Institute of Health AIDS Reagent Program

122787 Anti-Human CD3/4 Bispecific Monoclonal Ab lot no: 150084- National Institute of Health AIDS Reagent Program

The bispecific antibodies used for the VIA in collaboration from IAVI:

1. CD3/4 bi-specific monoclonal antibody (BSmAb): Prof J Wong, Harvard, USA.
2. CD3/8 bi-specific monoclonal antibody (BSmAb): Prof J Wong, Harvard, USA.

A panel of six viruses was used in this assay supplied by IAVI (Table 2.4). Three clade B viruses were used in the panel, one of which was laboratory grown (IIIB) and two were infectious molecular clones (IMC). Viruses from other clades were also used in order to provide information on cross clade viral inhibition. CH106 was provided from George Shaw (Perelman School of Medicine

University of Pennsylvania, USA). U455 and CBL4 were provided from the National Institute for Biological Standards and Control (NIBSC).

Table 2.4: Panel of viruses used in the viral inhibition assay. IIIB, CH077 and ELI were obtained from the National Institute of Health. CH106 provided from George Shaw (Perelman School of Medicine, University of Pennsylvania). U455 and CBL4 provided from the National Institute for Biological Standards and Control (NIBSC).

VIRUS	Culture	Clade	Tropism	Region
IIIB	Lab adapted	B	X4	United States
CH077	Infectious molecular clone	B	R5	United States
CH106	Infectious molecular clone	B	R5	United States
ELI	Lab adapted	A/D	X4	DR Congo
U455	Lab adapted	A	X4	Uganda
CBL4	Lab adapted	D	X4	Tanzania

Assay media

R10 and R20 media was prepared in-house in the same way and with the same reagents as in the T cell ELISpots

Other materials used in the VIA

Sterile Dulbecco's PBS without calcium or magnesium (Sigma D8537).
Complete RPMI with 10% fetal calf serum (medium for assay, R10, Appendix F).
Interleukin 2 (IL-2): supplied at 10 units per μL (Roche 1147528).
Sterile tissue culture water (Sigma W3500).
Virkon solution (VWR 220015406 or 115-0020): 2% w/v diluted fresh with tap
HIV-1 p24 ELISA: Perkin Elmer cat number NEK050

2.3.2 Optimisation of the assay

As we were unable to get the original bispecific antibodies used in the publication by Spentzou et al [201] from J.W Harvard Medical School, a new batch of bispecific antibodies was ordered from the National Institute of Health AIDS Reagent Program. The ability of these antibodies to generate pure

CD4+ and CD8+ populations needed to be assessed and conditions optimised. The extensive optimisation procedure is included in the appendix (Appendix 4).

With ongoing inadequate generation of pure populations of both CD4+ and CD8+ cells with the batch of bispecifics (although adequate expansion of cells up to 10 fold) , a decision was made to contact Dr Peter Hayes at the International AIDS Vaccine Initiative (IAVI) for further advice as they had recently conducted the VIA on another trial. Fortunately Dr Haye's team were using bispecific antibodies from the original paper with sufficient generation of pure cultures (>90%), and therefore a decision was made to conduct the VIA in collaboration with IAVI. This was conducted by me (and supervised by Dr Hayes and Dr Spentzou at IAVI) in the following way. Full methods have been previously described [201].

2.3.3 Viral Inhibition Assay Methods (VIA)

Time points assessed

Two time points were compared for the presence of viral inhibitory activity: Week 0/Visit 2 (prior to vaccination) and the primary end point Week 14/Visit 10 (two weeks after the third and final vaccination). Therefore for each participant, there was generation of one culture of pre vaccination CD4+ T cells and two CD8+T cell cultures (one pre and one post vaccination). This enabled direct comparison between the T cell ELISpot data and the viral inhibition assay data from the primary end point.

Thawing of cells and generation of CD4+ targets and CD8+ effector cells

In order to avoid weekend work, cells were thawed on a Tuesday, with all media and labelling of samples prepared in advance. Frozen PBMCs were removed from the freezer and thawed as described previously in a waterbath at 37°C. The medium used for this assay was R10.

Prior to centrifuging the cells, the required volume was removed for viable cell counting using the Vi-cell counter. Whilst counting, PBMCS were centrifuged at 250*g* at room temperature for 10 minutes. The supernatant was then decanted and the tube tapped to loosen the cell pellet. PBMCs were adjusted to 0.8-1.5million viable PBMC/ml R10.

For the generation of the CD4+ target cells, 6mls of PBMC were added to a T75 flask with CD3/8+ bispecific Ab (stock concentration 50µg/ml, final concentration 0.5µg/ml) and 30µL IL-2 (stock concentration 10 units/µL, final concentration 50 units/ml). The flasks were carefully mixed.

For the generation of the CD8+ effector cells, 3mls of PBMCs were added to a smaller T25 flask with CD3/4+ Ab (same concentration above) and 15µL IL-2 and carefully mixed. All flasks were incubated in the upright position at 37°C/5% $C0_2$.

On day 3 the starting volume was doubled by adding 6mls of R10/50 to the T75 flasks or 3mls to the T25 flasks. Flasks were placed back into the incubator but this time incubated upright to prepare for the further expansion of cells.

On day 6 all flasks were inspected microscopically for evidence of contamination and discarded if present. The culture volumes were again doubled with R10/50 (12 or 6mls respectively) and flasks incubated overnight upright ready for infection the next day. All labelling was carried out in preparation for the following day.

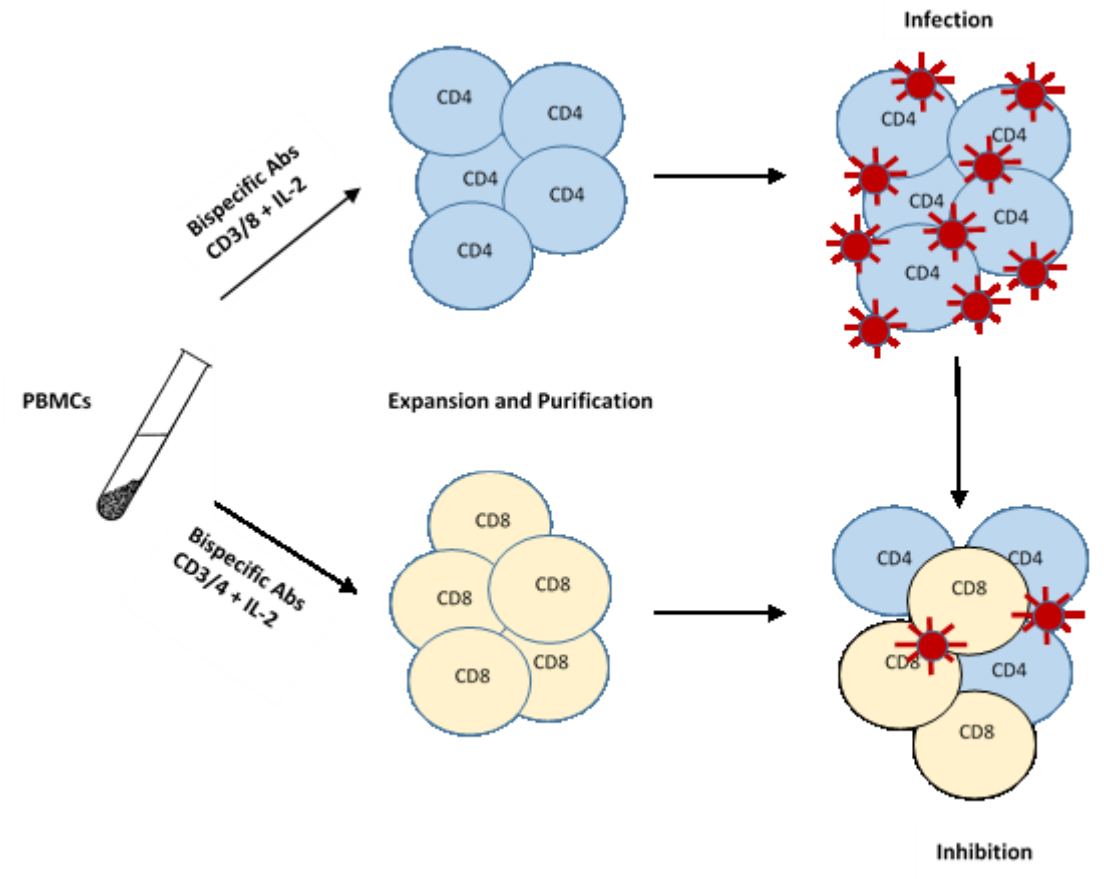


Figure 2.3: Graphic demonstration of VIA procedure

Day 7: Day of Infection

CD4+ cells (using CD3/8+ bispecific Abs for expansion and purification) were processed first to allow for a 4 hour infection period. Expanded CD4+ cells in the T75 flasks were resuspended by repeated

aspiration with a 10ml pipette before transfer to 50mls tubes and performing a viable cell count using the Vi-cell counter. Cells were not spun prior to counting during this step.

Once counting was complete, 0.5 million CD4+ cells per virus (plus an additional 0.2million cells) were added to 15ml tubes. The tubes were centrifuged at 250*g* for 10 mins and the supernatant decanted. The next steps were conducted in the Containment Level 3 (CL3) laboratory at IAVI.

CD4+ cells were infected with each HIV-1 virus at a multiplicity of infection rate (MOI) of 0.01 ie 10000 viral particles added per million CD4+ cells. The tubes were briefly vortexed to ensure adequate mixing of cells plus virus, and incubated for 4 hours at 37°C/5% $C0_2$ with loose caps.

Recovery of CD8+ cells

During the 4 hour infection period of the CD4+ cells, CD8+ cells in the T25 flasks were resuspended by repeated aspiration using a sterile pastette, and then recovered and counted as per CD4+ cells. CD8+ cells, R10 and sterile PBS were then added to a 48 well plate and incubated in preparation for the end of the 4 hour CD4+ infection period.

Once the CD4+ cells had completed the 4 hour infection incubation period, cells were washed and centrifuged with R10, decanted and resuspended with 1ml R10/IL-2 per million CD4+ cells (to achieve 100 units IL-2 per ml. For each virus tested the following cells were added resulting in a final IL-2 concentration of 50 units per ml.

0.5ml (0.5 million) CD4+ cells plus 0.5ml R10

0.5ml (0.5 million) CD4+ cells plus 0.4ml R10 plus 0.1ml (0.5 million) CD8+ cells

Post infection

On D3, D6, D9 500µl of supernatant was removed and the same volume fresh R10/50 added. The supernatant was discarded into virkon at these time points as it was not required for analysis. However, on D13 post infection, plates were examined microscopically in preparation for removing and freezing supernatant. 15µl triton per well was initially added to a 96 well plate followed by 135µl supernatant. Duplicate plates were conducted and frozen in different freezers (in case of freezer failure). The plates were wrapped in cling film to avoid them drying out. The plates were then ready for analysis using p24 ELISA described below.

P24 ELISA

This was conducted as follows using the HIV-1 p24 ELISA kit:

1. The p24 standard curve was made up to an upper concentration of 4000pg/ml by adding 20µl of p24 positive control to 980µl R10, and then 2 fold serially diluted down to 3.9pg/ml
2. Next 100µl R10 and p24 solutions were added to the required wells, followed by 100µl of sample dilution (samples made up to a dilution of 1/100 and 1/1000 for CD4+ wells and 1/10 and 1/1000 for all other wells)
3. The plates were incubated for 2 hours at 37°C/5%$C0_2$
4. After the incubation period the well contents were decanted gently into virkon and washed 6 times with wash buffer (made up by adding 26mls concentrate to 500mls sterile water)
5. Next 100µl detector antibody was added to all wells and incubated for a further hour at 37°C/5%$C0_2$
6. Wash steps repeated as above after the hour incubation
7. Prior to use, 140µl Streptavidin-HRP concentrate was added to a 14mls Streptavadin-HRP diluent (for each plate)
8. Next 100µl diluted streptavidin-HRP was added to each well and incubated at room temperature for 30 minutes. The plates were again washed as above after 30 minutes
9. Just prior to use, 1 OPD tablet was added to 11ml substrate diluent (per plate), and stored in the dark until ready to use. Once the tablet was fully dissolved and mixed well 100µl was added to each well making sure to discard if yellow or orange in colour as this suggested contamination.
10. The plates were incubated for 8-12 minutes at room temperature in the dark. Well contents were not washed or discarded after this step
11. Finally 100µl stop solution was added to all wells (4M sulphuric acid) and the plates were read at standard settings of 492nm against a reference of 620nm. This was done on the same day as the ELISAs were performed (usually within 15 minutes of stop solution being added)

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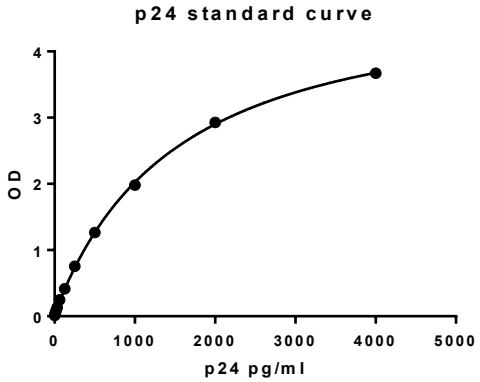


Figure 2.4: A typical p24 standard curve from the CUTHIVAC 001 trial data

2.3.4 Statistical analysis

Cut-off values for defining positive responses were determined by statisticians at EMMES Corporation using data accumulated from HIV negative subjects (including unvaccinated clinical trial participants) for all viral isolates tested. Cut-offs were defined by the 97.5th percentiles estimated using PROC QUANTREG in SAS 9.2. The overall 97.5th percentile (and 95% confidence interval) was defined as 1.5 $\log_{10}$ inhibition (1.43 – 1.60), reported by EMMES, February 3rd 2011.

The VIA response for each virus was defined as positive if the criteria below were fulfilled:-

a) The $\log^{10}$ inhibition is greater than 1.5
b) The difference between the post-vaccination and pre-vaccination response should be $\geq$0.6 $\log^{10}$ inhibition

All statistical analysis was conducted using *Graphpad prism version* 6.0. The mean, median and maximum $\log^{10}$ inhibition was calculated for each group and a one-way ANOVA for-non parametric data was used to analyse differences between the three groups.

To analysis whether there was a difference in $\log^{10}$ inhibition for each group at week 14 (primary end point) compared to week 0 (pre-vaccination) a paired t test (Wilcoxon matched-pairs signed rank test) was used. A result was considered statistically significant if the 2 tailed p value was <0.05.

Finally, to analyse whether there was a correlation between the VIA results and the IFN-ɣ ELISpot results, I conducted a correlation analysis using *Graphpad prism*, and calculated the R^2 correlation coefficient.

2.4 CUTHIVTHER 001: STUDY SUBJECTS AND VACCINE SPECIFICS

The CUTHIVTHER 001 was a Phase I/II randomised controlled clinical trial to investigate the safety and immunogenicity of the same GTU®MultiHIV B clade DNA vaccine administered 3 times in people living with HIV who were stable on antiretroviral therapy (ART). The vaccine was administered either intramuscularly (IM) and enhanced with EP, or IM with TC delivery. In this trial there was the inclusion of a placebo group in anticipation of the potential for high background responses to peptide pools in an HIV-infected cohort of participants.

2.4.1 Study Participants

All study visits were conducted at the Clinical Trials Centre, St Mary's Hospital London. HIV infected participants who had been on ART for at least 6 months were actively recruited from the Wharfside clinic (St Mary's Hospital) and HIV clinics linked to Chelsea & Westminster Hospitals. The main inclusion and exclusion criteria are shown in the table below.

Table 2.5. Inclusion/exclusion criteria CUTHIVTHER 001

INCLUSION CRITERIA	EXCLUSION CRITERIA
Men and women aged between 18-45	Pregnant or lactating
BMI between 19-30	Use of topical treatment on injection or application site within last 4 weeks
Willing and able to given informed written consent	No UV tanning sessions or strong sun exposure within 4 weeks prior to study
HIV Clade B infection documented by confirmed Ab test	Clinically relevant abnormality on history or examination including: • History of seizure disorder • Severe eczema • Liver disease including active Hep B (HepBsAg pos) or C (PCR positive) • Any skin condition which may interfere with injection site • Haematological, metabolic, gastrointestinal, cardiac disorders including abnormal ECG
Confirmed on 2 separate occasions in the 6 months prior to enrolment to have viral load <50 copies HIV RNA/ml whilst on ART	
Nadir CD4+ ≥250 and screening CD4+ ≥200 lymphocytes	Excessive terminal hair growth on investigational areas
	Grade 2 or above abnormal laboratory parameters (including conjugated hyperbilirubinaemia)
If heterosexually active female using an effective method of contraception (includes consistent condom use). If heterosexually active male using effective method with partner	Previous severe reaction to vaccination or receipt of live attenuation vaccine in last 60 days

2.4.2 Ethical approval

The CUTHIVTHER 001 trial was conducted in compliance with UK Clinical Trial Regulations and within the principles of Good Clinical Practice (GCP). The study was approved by the National Research Ethics Service, York North East Research Ethics Committee and by the Medicines and Healthcare products Regulatory Authority (MHRA). All participants provided informed written consent after thorough counselling by me as co-investigator on this trial.

2.4.3 Vaccine candidate and administration

The same investigational GTU®MultiHIV B clade DNA vaccine was used as in the previous trial.

In this trial however, participants were randomised into one of 2 groups (either EP+IM or TC+IM) with a further randomisation to receive placebo or vaccine (Table 2.6). A placebo group was deemed necessary in this trial in anticipation of high pre-vaccination responses to the peptides in an HIV infected cohort. An ID+IM group was excluded in this trial, based on few immune responses from the previous trial

All vaccinations were conducted in the same manner as in the previous trial at weeks 0, 4 and 12. However, based on our trial team experience of pain associated with receiving electroporation to both legs in the CUTHIVAC 001 trial (see results section), only half the dose was administered to each participant in the EP+IM group (ie one leg only receiving 2mg/1ml vaccine or placebo immediately followed by EP). In the TC+IM group, TC vaccine administration was administered to one arm as in the previous trial (0.4mg/0.2mls), with IM vaccination (2mg/1ml) administered to one leg only. If a participant was randomised to the placebo group normal saline was administered instead of the vaccine. The participants remained blinded throughout the trial.

Table 2.6: Vaccine schedule for CUTHIVTHER 001 including groups, route of vaccination and cumulative dose of DNA. Placebo = normal saline.

Group	Route and Vaccine Schedule			Cumulative dose DNA
	Week 0	Week 4	Week 12	
1.EP+IM (n=15) 10 Active 5 Placebo	2mg IM GTU MultiHIV DNA	2mg IM GTU MultiHIV DNA	2mg IM GTU MultiHIV DNA	6.0mg (active) 0.0mg (placebo)
2.TC+IM (n=15) 10 Active 5 Placebo	2mg IM 0.4mg TC GTU MultiHIV DNA	2mg IM 0.4mg TC GTU MultiHIV DNA	2mg IM 0.4mg TC GTU MultiHIV DNA	7.2mg (active) 0.0mg (placebo)

2.4.4 Reactogenicity and safety

As there were no safety concerns from the previous trial, participants were only required to attend for the 7 day safety assessment in this trial, and the 24 hour visit was replaced by a telephone call. Participants filled out diary cards from 12 hours to 7 days post vaccination as in the previous trial. Those randomised to EP+IM were asked to fill out a pain questionnaire rating their experience of the procedure, using a standardized pain score (0=no pain, 10=worst pain ever), and also conducted an acceptability questionnaire relating to the procedure.

2.4.5 Human leucocyte antigen (HLA) typing

Blood was taken at week 0 and high and low resolution HLA class I typing was performed by sequence specific PCR according to standard protocols at the Department of Histocompatibility and Immunogenetics, Hammersmith Hospital. The final HLA I typing result is presented as an 'interpreted' HLA type combined high and low resolution PCR.

2.4.6 Immunogenicity sampling schedule and PBMC processing

Blood samples were collected from participants at the same specified time points as in the previous trial, with PBMCs processed in the same manner. Serum samples were also collected for CD4+ count and HIV viral load at the same specified time points and process in the immunology and virology labs at St Mary's Hospital.

2.5 CUTHIVTHER 001 IMMUNOGENICITY: T CELL IFN-ɣ ELISPOT ASSAY

2.5.1 Reagents and buffers

To ensure consistency of results across both trials all reagents and buffers used were the same as in CUTHIVAC 001. Additional reagents are listed below.

Integrase peptide pool

HIV-1 Consensus B Env peptide pool obtained from NIH-AIDS

Catalog No: 12540; Lot No: 140347; Expiry: 12/08/16

2.5.2 IFN-ɣ ELISpot Assay

Optimisation of integrase pool

T cell immunogenicity was assessed by IFN-ɣ ELISpot assays on frozen PBMCs stimulated by peptide pools matched to the GTU®MultiHIV B vaccine as in the previous trial. An additional integrase peptide pool was added as a control to the assay as the presence of a peptide pool not included in the vaccine may give information on any non-specific changes occurring within T cells related to vaccination. Previous data mapping of CD8+ responses to the integrase peptide pool in 56 infected subjects showed 68% of subjects recognized epitopes within the integrase peptide pool (using ELISpot assays measuring IFN-ɣ), with no significant differences in T cell response comparing acute and chronic infection [202].

In order to optimise this additional peptide pool, the integrase sequence was ordered in its entirety from NIH-AIDS. The relevant integrase peptides were then pooled together. The concentration of the integrase peptide pool used in the assay was 10μg/ml, with a final concentration in each well of 2.5μg/ml as per the other peptide pools.

The T cell ELISpot as described previously was conducted on 8 HIV uninfected donor cones in order to assess the background response to the integrase peptide pool. Apart from Donor 23 which showed a high background response and is therefore excluded from analysis, all others donors had a negative response as expected (<55 SFU/M PBMC). All responses are presented as SFU/M PBMC with background subtracted (Table 2.7). CEFT and PHA were again used as positive controls in this optimisation assay.

Table 2.7: Optimisation results from the integrase peptide pool in 8 donor cones. All results are expressed as SFU/M PBMC.

	Integrase	**CEFT**	**PHA**
Donor 14	0	2	4683
Donor 21	0	73	12500
Donor 22	0	95	7367
Donor 23	127	47	19383
Donor 24	0	15	7483
Donor 25	0	185	7550
Donor 26	25	315	15717
Donor 28	5	107	6750

Addition of the integrase peptide pool

The additional integrase pool was used in the ELISpot assay at the following time points: Week 0 (pre-vaccination), Week 14 (primary end point, 2 weeks after third and final visit), and Week 20 (final follow up visit). Therefore for these visits the plate plan has been modified (Figure 2.5). All other visits were conducted as in the previous trial following the same methods for the ELISpot assay and using the same plate plan previously displayed.

1	2	3	4	5	6	7	8	9	10	11	12
NEG			NEG			NEG			INT		
REV			REV			REV					
TAT			TAT			TAT					
NEF			NEF			NEF			INT		
GAG			GAG			GAG					
CTL			CTL			CTL					
PHA			PHA			PHA			INT		
CEFT			CEFT			CEFT					

Figure 2.5: Plate plan for Weeks 0, 14 and 20 denoting presence of integrase peptide pool.

Definition of a 'positive' response

In this trial the definition of a positive response was modified due to the likelihood of high and variable baseline responses to peptide pools in this cohort of participants living with HIV. Therefore a positive response was defined as a doubling in frequency of IFN-ɣ SFU/M PBMC from baseline, or the presence of a response at the primary end point (week 14) or other specified time points that was absent at baseline.

2.5.3 Statistical Analysis

All data was analysed using *Graphpad prism* version 6.0. As in the previous trial, the primary end point was defined as 2 weeks after the third and final vaccine (week 14).

The difference in magnitude of the INF-ɣ response between the groups at the specified time points including the primary end point for each peptide was compared using an unpaired t test (non-parametric Mann-Witney test). A result was considered statistically significant if the 2 tailed P value was <0.05.

To analyse the difference within each individual group between week 0 and week 14, a paired t test (Wilcoxon matched-pairs singed rank test) was used with statistical significant set at p=<0.05.

2.5.4 Supporting work: Intracellular cytokine staining (ICS)

T cell responses were further evaluated by multi-parametric ICS with two 11 colour panels, one for CD8+ and one for CD4+ according to standard protocols.

The ICS assays were conducted by Celine Yan and Suzanne Venables (Research Technicians, Shattock group, Department of Medicine Imperial College London).

The ICS data is presented as a percentage total of CD4+ or CD8+ cells with all background responses from negative controls subtracted. For the CD4+ results data for cytokines IFN-ɣ, IL-2, TNF-α and CD154 is presented, and for CD8+ cytokines IFN-ɣ, IL-2, TNF-α, CD57, granzyme B and perforin.

Although I did not conduct the ICS practical procedure, I did conduct an analysis of the data and it is important to include this data when interpreting the T cell immunogenicity results. The analysis was conducted using *Graphpad Prism* version 6.0.

2.6 CUTHIVTHER 001: Viral Inhibition Assay

Although the VIA has been conducted in a limited number of HIV positive people, the assay has only been validated to assess *in vitro* CD8+ mediated inhibition in HIV uninfected subjects. Previous literature has shown in HIV positive subjects not on ART but with low viral loads (<10,000 copies/ml), there is evidence of inhibition which is not displayed in those with higher viral loads [201]. There are limitations with this assay when applying it to HIV positive subjects, including the inability to distinguish between endogenous and exogenous virus in the final p24 reading.

As the participants in CUTHIVTHER 001 were HIV infected, the assay required a new approach and modification for an HIV infected cohort. Two main questions need to be considered:

1. **Can the CD8+ cells of vaccinated participants in the trial inhibit their endogenous virus?** This would involve a modification of the VIA where CD4+ cells would not be infected with exogenous virus as per standard assay, but p24 readings pre and post vaccination would be analysed to answer whether endogenous virus was inhibited.

2. **Can the CD8+ cells of vaccinated participants in the trial inhibit exogenous virus?** To answer this question prior to CD4+ infection with exogenous virus, endogenous virus will first need to be blocked by adding antiretroviral/s to the cultures, to ensure final p24 reading are from exogenous virus replication only.

As this was a new approach to the assay, I set up a meeting with my supervisors and the team at IAVI. We decided the CCR5 inhibitor Maraviroc (MVC) could be a suitable drug to be utilised in the assay as it has been used extensively in tissue culture assays with minimal toxicity. MVC was approved by the US Food and Drug Administration (FDA) in 2007 and is now utilized as an antiretroviral agent in HIV positive individuals who are R5 tropic [203]. Although the clinical course of HIV is highly variable between patients, it is thought CCR5 viruses predominate in early HIV infection with a shift to CXCR4 viruses occurring much later in the course of HIV infection correlating with decreases in CD4+ count and disease progression [204]. As the cohort of HIV participants in this trial were generally healthy with nadir CD4+ counts >250, maraviroc was deemed suitable under the assumption the cohort of trial participants were R5 tropic.

In order to assess the suitability of MVC, an optimisation experiment was conducted at IAVI with results shown below.

2.6.1 Reagents and buffers

To ensure consistency of results across both trials all reagents and buffers used were the same as in CUTHIVAC 001. Additional reagents/materials are listed below

Maraviroc

Supplied by Dr Carolina Herrera, Imperial College London (initially obtained from Dr Clare McCoy of Queen's University Belfast School of Pharmacy for research purposes only).

20mM stock solution prepared in PBS. This was diluted 1/10 in PBS and this was then filtered using a 0.2 µm filter. 5µL of this 2mM solution was added per mL (1/200 dilution giving a final concentration of 10µM).

2.6.2 Maraviroc optimisation experiment

8 HIV positive donors with frozen PBMCs available at IAVI were selected for the optimisation procedure, 4 of which were on ART with low or undetectable viral loads with previous VIA conducted showing endogenous release of p24 from CD4 cells at approximately 100,000pg/ml. The other 4 subjects were not on ART with high viral loads (Table 2.8).

Table 2.8: Subjects used in Maraviroc optimisation assay with demographic information available.

	Donor	Sample date	Clade	CD4	VL
Subjects with low VLs on ART	C624	13/11/2006	B	347	<50
	H110	14/11/2006	A/G	312	57
	L200	15/11/2006	B	348	<50
	M325	23/11/2006	B	359	<50
Subjects with high VLs not on ART	G420	01/04/2008	NK	978	87567
	W340	14/08/2007	NK	666	50187
	S530	18/04/2008	NK	304	69410
	M160	25/01/2010	NK	658	2246

3 cultures/conditions were then set up and the VIA conducted following the previous steps. The CD4+ T cells were generated by 7 day expansion with CD3/8 bispecific antibodies and IL-2 with the media doubled at day 3 and 6 as per previous assay. The cultures included are listed below.

- Culture 1**:** No Maraviroc
- Culture 2**:** 10uM Maraviroc and add fresh R10/50 with 10uM Maraviroc at day 3 and 6
- Culture 3**:** 10uM Maraviroc and add fresh R10/50 with 20uM Maraviroc at day 3 and 6 (the concentration was doubled in this culture assuming the drug may degrade over a few days)

After 7 days the expanded CD4+ cells were washed and no further drug was added in the cultures. Plates were set up as per VIA, but no exogenous virus was added, therefore only endogenous growth was examined by p24 measurements. P24 content in supernatant was analysed both at day 13 as per usual assay, but also out to day 20.

The results showed the following (Figure 2.6):

- 7 subjects exhibited endogenous virus (all but C624)
- In most of the subjects not on ART, Maraviroc reduced endogenous virus

- In subjects H10 and L200 the endogenous virus was not blocked by Maraviroc- there is no data available on whether these subjects were R5 or X4 tropic.
- At day 20 there was more p24 than day 13

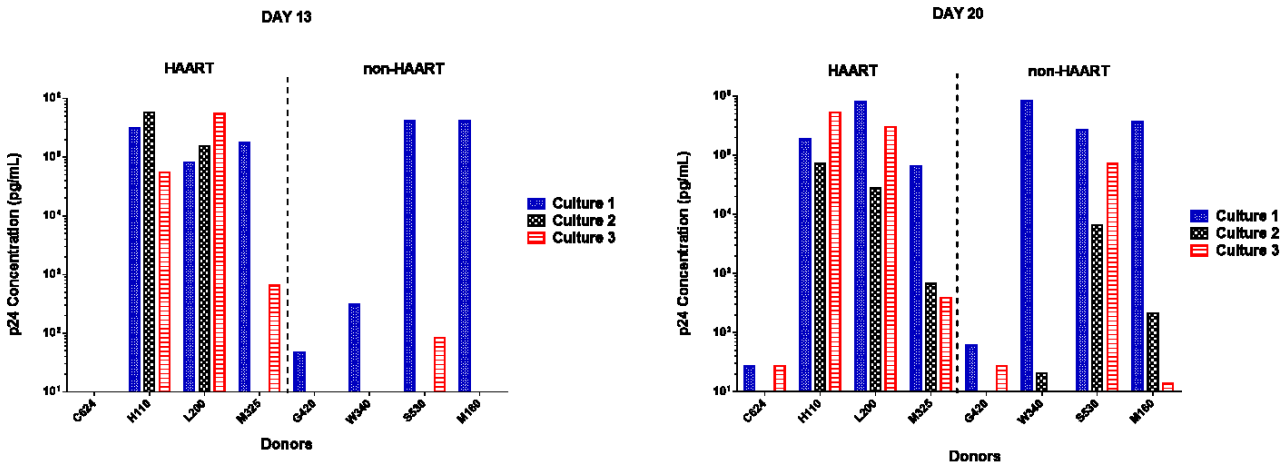


Figure 2.6: p24 concentration (pg/ml) at Day 13 and Day 20 (cultures continues for additional 7 days)

To conclude this optimisation process, Maraviroc was safe and non-toxic to the growth and expansion of cells, and did seem to inhibit endogenous virus in most subjects. We therefore confident this would be an appropriate choice of antiretroviral to continue with the VIA.

2.6.3 VIA methods to address inhibition of endogenous virus (Question 1)

1. Can the CD8+ cells of vaccinated participants in the trial inhibit their endogenous virus?

For this part of the assay, PBMCs from Week 0 and Week 14 for each participant were thawed as per previous with CD4+ targets and CD8+ effectors generated using the same steps (involving bispecific antibodies and IL-2).

At D7, both CD4+ and CD8+ cells were recovered. The CD4+ cells were not infected with exogenous virus. A 48 well plate was set up with 0.5million CD4 cells alone pre vaccination (week 0), 0.5milion CD4 cells post vaccination (week 14), and CD4+ and CD8+ cells pre and post vaccination. As in the previous assay, on days 3, 6 and 9 supernatant was removed and replaced with fresh R10/50 media. On day 13 supernatant was removed and frozen in duplicate ready for p24 ELISA analysis. The readings from this assay therefore represent the difference between endogenous virus pre-vaccination and at the primary end point as no exogenous virus was added. The change from the

previous assay is demonstrated below schematically where X indicates the step omitted in this assay (Figure 2.7).

For the p24 ELISA, all samples for Week 0 and Week 14 from participants were screened for the presence of endogenous virus, and only in those with virus at either time point was the ELISA conducted as per previous methods.

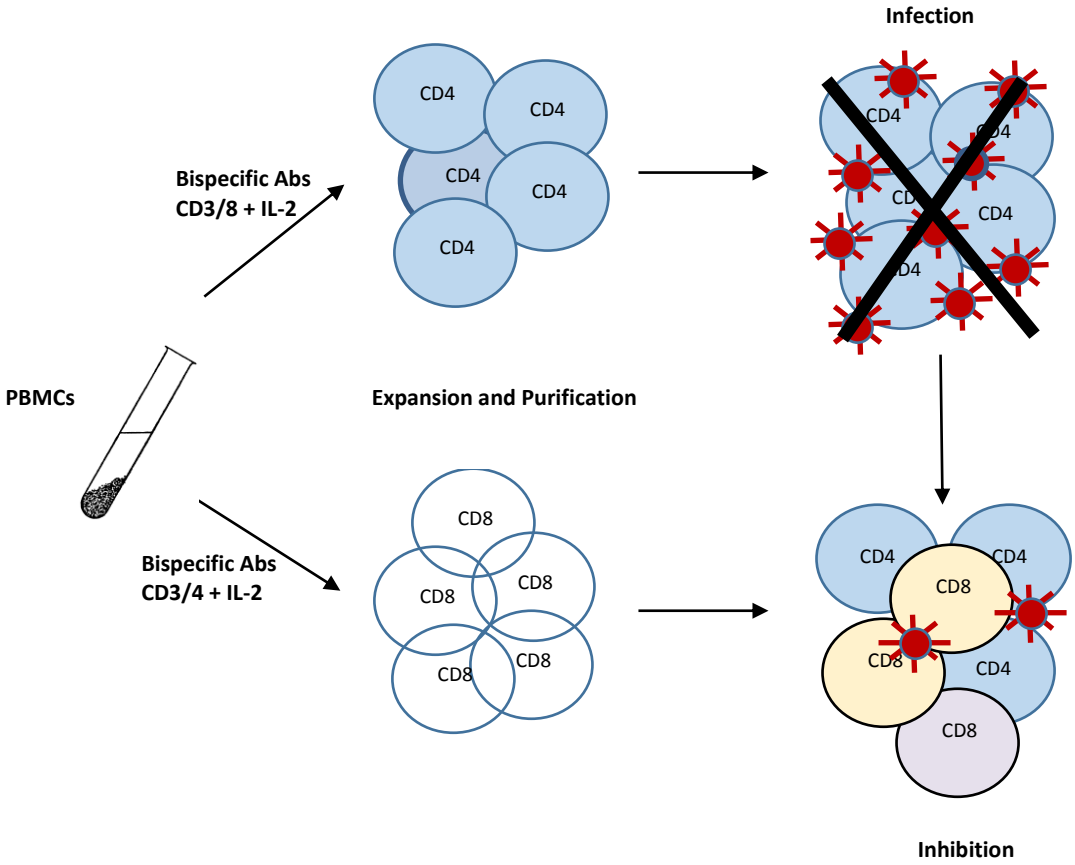


Figure 2.7: Adapted VIA where no exogenous virus is added.

2.6.4 VIA methods to address inhibition of exogenous virus (Question 2)

2. Can the CD8+ cells of vaccinated participants in the trial inhibit exogenous virus?

In order to address this question, I conducted the following adaptations to the original assay. The same PBMC vials used above from each participant at Week 0 and Week 14 were expanded and cultured for 7 days with the bispecific antibodies and IL-2. However, MVC at a concentration of 10µM was added into the culture and on days 3 and 6 when the culture volume was doubled, in order to target any endogenous virus. A schematic of the assay is shown below with the inclusion of MVC (Figure 2.8).

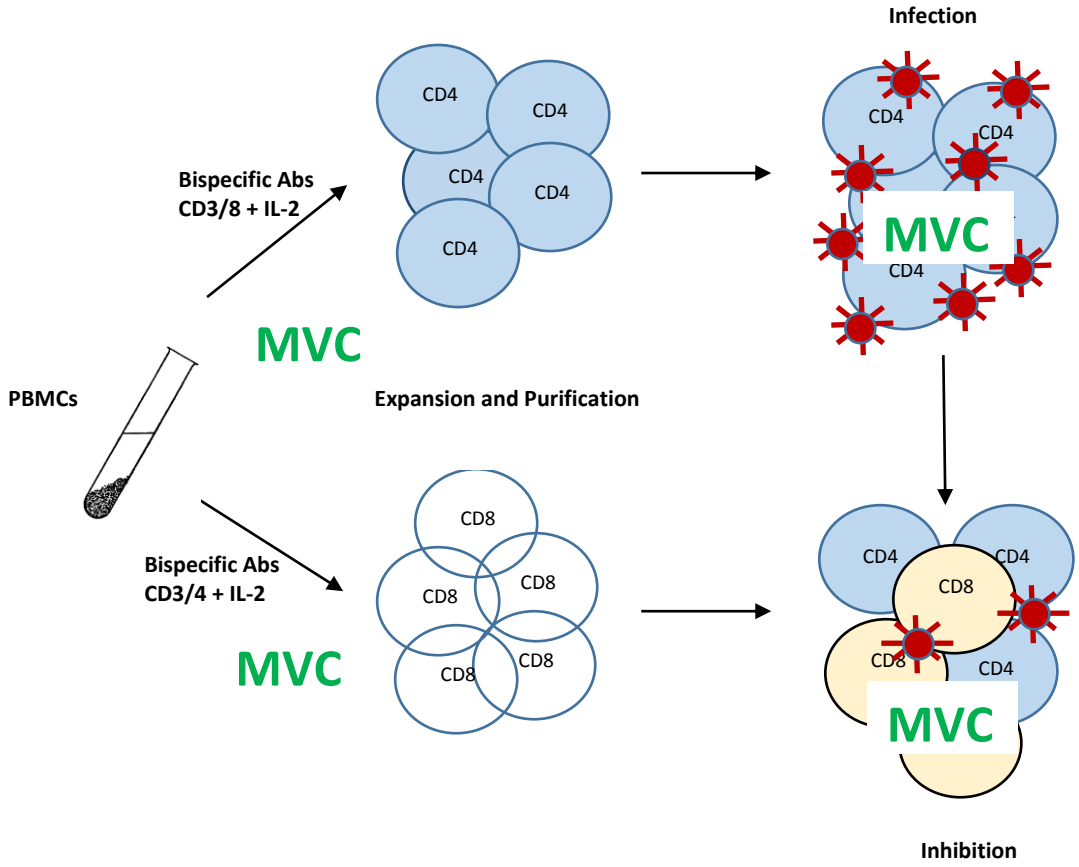


Figure 2.8: Adapted VIA where MVC is added throughout the assay to block any endogenous virus production

At day 7, the cells were infected with exogenous virus. Due to cell number and availability, 3 viruses only were used and these were X4 viruses to prevent MVC (an R5 inhibitor) from blocking added virus (Table 2.9).

Table 2.9: Modified panel of viruses for CUTHIVTHER 001

VIRUS	Culture	Clade	Tropism	Region
IIIB	Lab adapted	B	X4	United States
U455	Lab adapted	A	X4	Uganda
CBL4	Lab adapted	D	X4	Tanzania

The assay continued as per previous methods, the only difference being MVC continued to be added to the wells when supernatant was replaced with fresh medium (R10/50) in order to block any replication of endogenous virus. The p24 ELISAs were conducted using the same methods as previous.

2.6.5 Statistical analysis

All data analysis was conducted using *Graphpad prism*. For this assay as it has not been validated for this cohort, and I was anticipating potential inhibition at baseline, the cut-off of 0.6 $\log^{10}$ could not be used, but the cut-off for a positive response of >1.5 $\log^{10}$ inhibition for a positive response was carried forward.

To analysis whether there was a difference in $\log^{10}$ inhibition for each group at week 14 (primary end point) compared to week 0 (pre-vaccination) a paired t test (Wilcoxon matched-pairs signed rank test) was conducted. A result was considered statistically significant if the 2 tailed P value was <0.05.

Chapter 3
Results: Trial Data CUTHIVAC 001

3.1 INTRODUCTION

Although globally new HIV-1 infections have decreased worldwide without the development of an HIV prophylactic vaccine, key populations are still at risk with concerning numbers of new infections in regions such as Central Asia and Eastern Europe [205]. These considerations justify the ongoing pursuit of a HIV vaccine. However, few HIV vaccine efficacy trials have been conducted for a disease with such global importance. Factors include challenges such as the diversity of the virus, rapid integration into the host genome, and the establishment of latency [206]. Reassuringly however, vaccine trials that have progressed past phase I and II have generally been well tolerated with acceptable safety profiles.

Historically recruitment into phase I clinical trials has been challenging, and none more so than for HIV vaccine trials. Reported barriers to recruitment include time commitments, perceived risks of HIV infection from receiving a vaccine and stigma associated with HIV [207]. Motivational factors to take part can be psychological, physical or financial [208], and the ethics of receiving financial incentives are discussed later in this chapter in relation to motivation data collected from this trial.

CUTHIVAC 001 was designed to explore the safety and immunogenicity of different methods of delivery of the GTU® MultiHIV B clade DNA vaccine in HIV uninfected volunteers. With any phase I trial, the safety of the investigational product is paramount, and in this chapter trial data is discussed in detail including the recruitment of volunteers, demographics and safety data.

Although this was a phase I trial it was not 'first in man', and to date this vaccine has been evaluated in 28 uninfected volunteers (administered IM and ID), and 63 HIV infected volunteers (administered IM and ID and compared with placebo) with no serious safety concerns [135]. In both these trials the dose of DNA was 50% of that used in this trial (2mg), thus it is important to note the vaccine has not been investigated at the higher dose of up to 4.4mg DNA per vaccine visit in CUTHIVAC 001.

The primary safety endpoint was defined as the presence of a grade 3 or above local or systemic solicited adverse event or an adverse event that resulted in a clinical decision to discontinue further immunisations. All adverse events were graded according to the Food and Drug Association (FDA) toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventative vaccine

trials (Appendix 5). Data on safety events were recorded from all visits as well as from participant diary cards. The vaccination schedule is shown in the figure below (Figure 3.1). Although my hypotheses were based on the immunogenicity of the different routes of DNA delivery, the documentation and presentation of safety data is important particularly when investigating novel techniques of vaccine delivery.

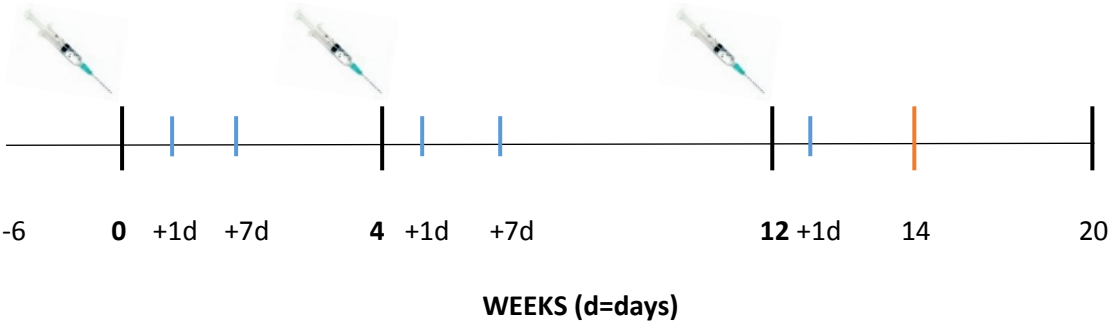


Figure 3.1: Diagram to show the 3 vaccination time points at weeks 0, 4 and 12. The primary end point is shown in orange at week 14, and week -6 denotes the 6 week period between screening and possible enrolment. After each vaccination there were 2 safety visits at Day 1 and Day 7 as indicated and the final follow up was at week 20. All groups followed the same vaccination schedule.

3.2 RESULTS

3.2.1 Participant Recruitment

Recruitment to this trial was challenging and participants were recruited using a variety of methods. Primarily the 'Help Make History' network of trial volunteers was used for recruitment (www.helpmakehistory.mrc.ac.uk). This is a trial specific network used to recruit HIV uninfected individuals into clinical trials. Recruitment using this avenue however was slow and therefore expanded to include setting up a CUTHIVAC facebook page, a blog account and a twitter account by our recruitment officer. Previous data has shown social media platforms to be highly successful both in disseminating information on trial results and helping trial recruitment [209].

Aside from digital platforms, 2 adverts were placed in the Metro/Evening Standard newspapers, a full page advert in the London Pride Guide event, and an advertorial in the Guardian Lifestyle Supplement (Figure 3.2). Social media platforms generated the most trial related enquiries which translated to the source of the greatest number of participants enrolled (Tables 3.1 and 3.2).

Figure 3.2: The Metro and Evening Standard advert used (top left); double page spread in London Pride Guide (top right); advertorial in 'The Scene' Guardian newspaper supplement (bottom left and right).

Table 3.1: Source of trial related enquiries and number of participant enquiries generated per source.

A: Source of enquiry	Number of people enquiring
Social media (Facebook, Twitter, Help Make History)	174 (Facebook 41, Twitter 60, HMH 73)
Metro/Evening Standard	168
London Pride Guide	13
The Guardian supplement	10
QX magazine	2
Friends/Family (word of mouth)	9
Other Centre	12
Gaydio (radio station)	3
GP referral	1
NHS choices	1
AIDS Map link on website	6
Not specified	21
Total	**420**

Table 3.2: Table to highlight 1/3 of participants enrolled were attracted to the study through social media (10/30 participants)

B: Source of enquiry	Number enrolled
Social media	10
Metro/Evening Standard	9
Friends/Family (word of mouth)	5
The Guardian Supplement	2
Other Centre	2
QX magazine	1
London Pride Guide	1
Total	**30**

3.2.2 Enrolment and participant flow

Enrolment occurred between April and November 2014. In total I screened 59 participants to enrol 30 eligible into the trial (screening to enrolment ratio 1.9:1). The main reasons participants failed screening were abnormal liver function tests above Grade 1 (>1.25 to 2 times the upper limit of normal), most commonly alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Figure 3.3). Other common exclusions included BMI >30 and cardiovascular exclusions related to the EP device, such as having a resting pulse rate <50 beats per minute. Skin related exclusions included eosinophilic folliculitis (a benign condition but potentially could decrease transcutaneous absorption of the vaccine), and excess terminal growth hair on the vaccine site, again related to the transcutaneous route.

In total 30 participants were successfully enrolled into the trial- 17 males and 13 females. The majority of participants enrolled were of White origin (77%) with a mean age of 30.7 years. Most participants were in full time employment (70%) (Table 3.3).

Data was also collected on reasons for entering the trial, where at screening I asked participants 'What motivated you to volunteer for this study? The main reasons for taking part were financial compensation (38%) and helping research (31%) (Figure 3.4). In this trial participants received a total of £1400 to attend all visits, a pre-defined and agreed compensation amount with the ethics committee responsible for this trial.

In total, 3 participants did not complete all vaccine visits but remained in follow up: in group 1 the participant moved away before the trial was completed and only received 2 vaccines; in group 2 the participant chose not to receive the third vaccine due to persisting hypopigmentation as a result of the TC procedure; in group 3 the participant chose not to continue with vaccinations due to pain as a result of EP after the first vaccination (Figure 3.5 Consort diagram).

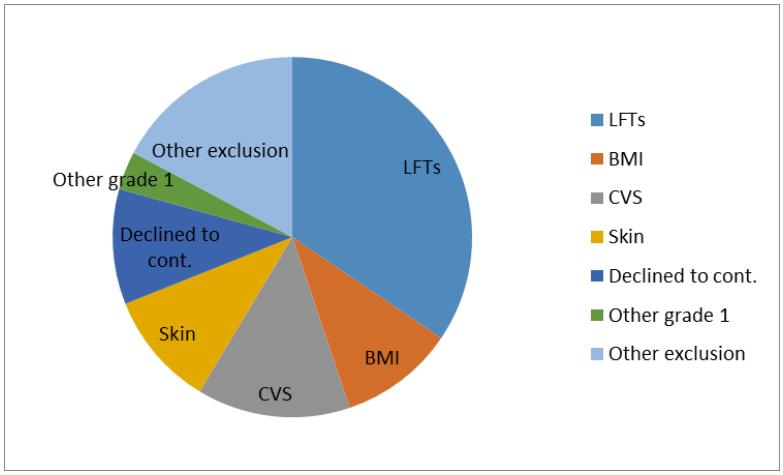


Figure 3.3: Pie chart showing reasons participants who were screened were excluded from the trial

LFTS: Liver function tests; BMI: Body mass index; CVS: Cardiovascular

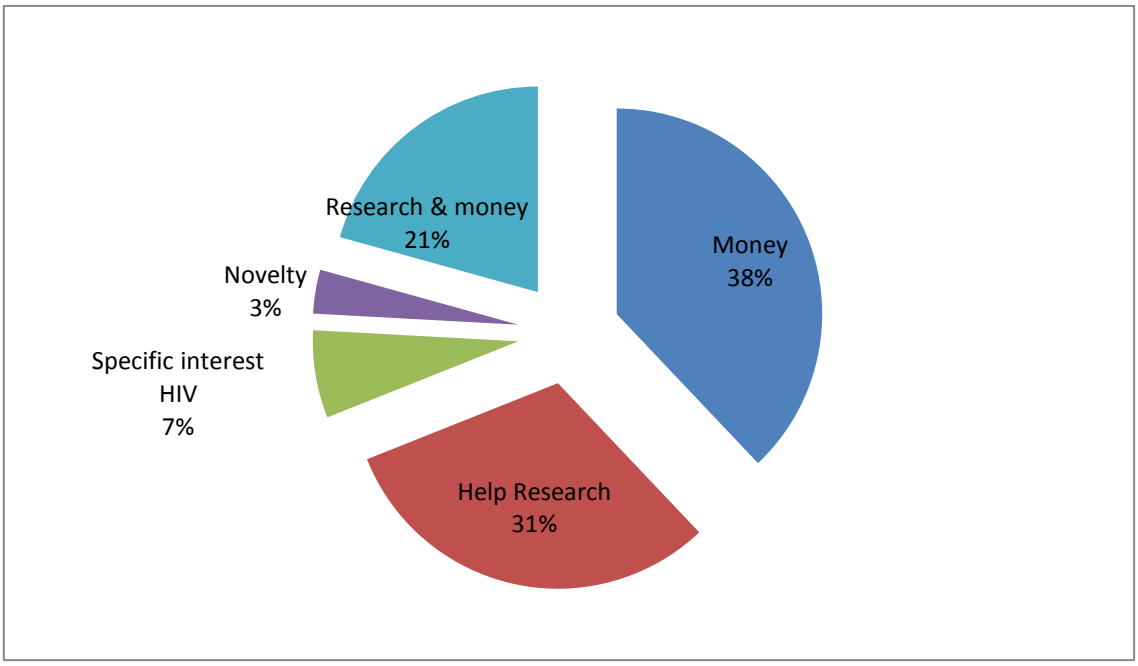


Figure 3.4: Pie chart to show participant motivation for entering study (n=30)

Table 3.3: Participant demographics (those who were enrolled. N=30)

Demographics	Group 1 (ID + IM)	Group 2 (TC + IM)	Group 3 (EP + IM)	Total
Gender				
Male	5	6	6	**17**
Female	4	5	4	**13**
Ethnicity				
White British	6	2	4	**12**
White Other	2	5	4	**11**
Mixed Ethnic Group	1			**1**
Black African		1	1	**2**
Black Other		1	1	**2**
Asian (Chinese)		1		**1**
Asian (Other)		1		**1**
Age (years)				
Mean	28.6	31.5	31.7	**30.7**
Range	(22-34)	(21-42)	(23-39)	**(21-42)**
Employment status				
Full time	7	7	7	**21**
Part time			1	**1**
Unemployed	1			**1**
Student	1	4	2	**7**

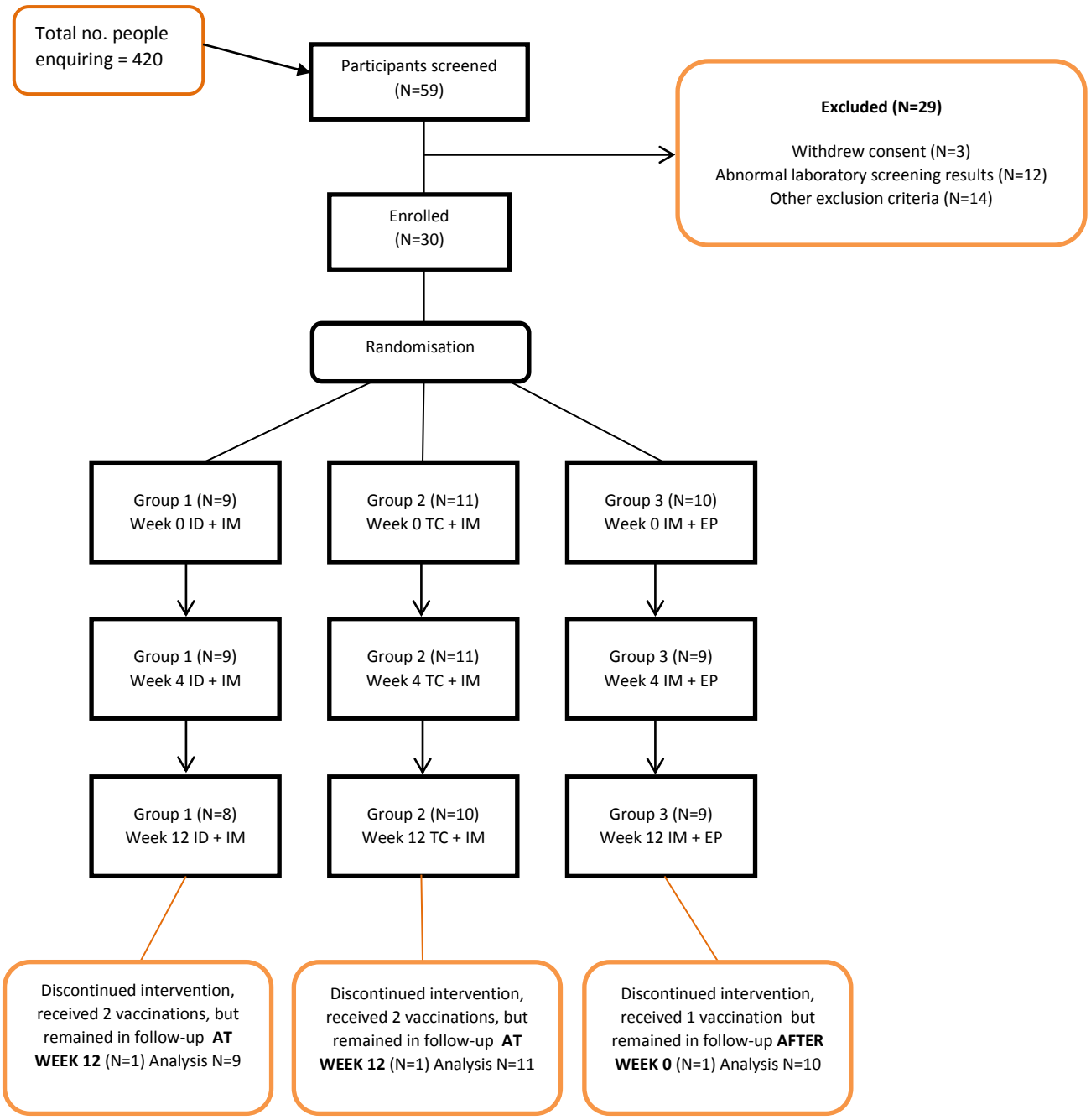


Figure 3.5: Consort diagram outlining participant flow in the 3 groups.

3.2.3 Safety and reactogenicity

Overall the GTU®MultiHIV B clade DNA vaccine was safe and well tolerated across all 3 vaccination groups. The majority of unsolicited adverse events were unrelated to the vaccination process based on the clinical team's judgement and safety end point review (Table 3.4), and there were no serious adverse events experienced throughout the trial.

From participant diary card entries, there were no statistically significant differences in general or local symptoms between the groups (Tables 3.4 and 3.5). All reactions were either grade 1 or grade 2 (mild/moderate) with no grade 3 or 4 reactions documented from participant diary cards (FDA toxicity table Appendix 5). The numbers documented represent the number of participants recording the symptom at any time point. Therefore if participants recorded the same symptom on every diary card, this is only counted once.

Table 3.4: Summary of unsolicited adverse events by group and relationship to vaccine

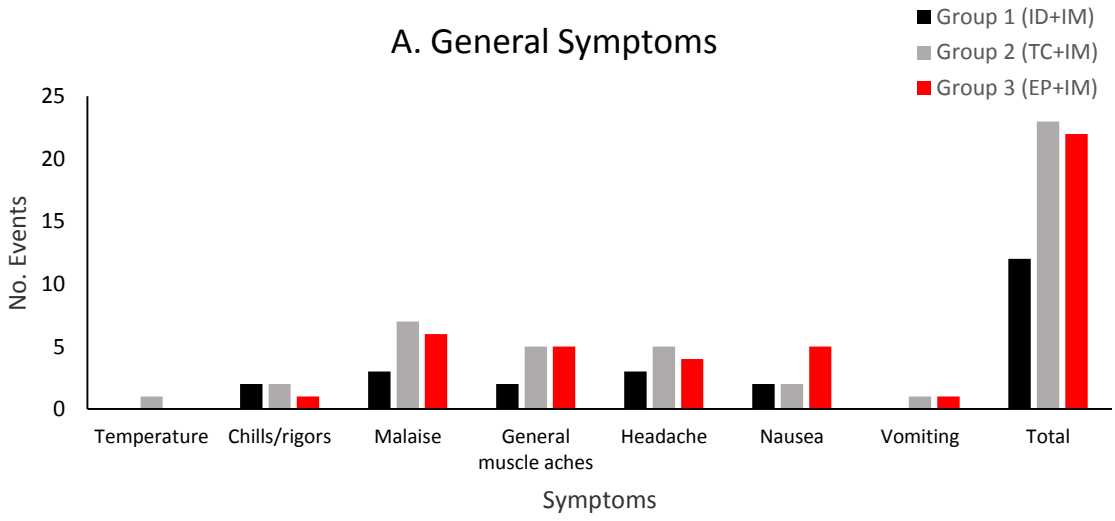
	Symptoms	ID+IM (Group 1) (n=9)	TC+IM (Group 2) (n=11)	EP+IM (Group 3) (n=10)
Systemic reactions (p=0.49)	Temperature	0	1	0
	Chills/Rigors	2	2	1
	Malaise	3	7	6
	General muscle aches	2	5	5
	Headache	3	5	4
	Nausea	2	2	5
	Vomiting	0	1	1
	Total	**12**	**23**	**22**
Local reactions Arm (p=0.69)	Pain	3	2	n/a
	Itching/irritation	2	6	
	Redness/discolouration	2	7	
	Lumpy swelling	1	0	
	Total	**8**	**15**	
Local reactions Leg (p=0.03 ID vs EP) (p=0.06 TC vs EP)	Pain	2	3	10
	Itching/irritation	0	2	3
	Redness/discolouration	2	2	5
	Lumpy swelling	1	0	4
	Total	**5**	**7**	**22**

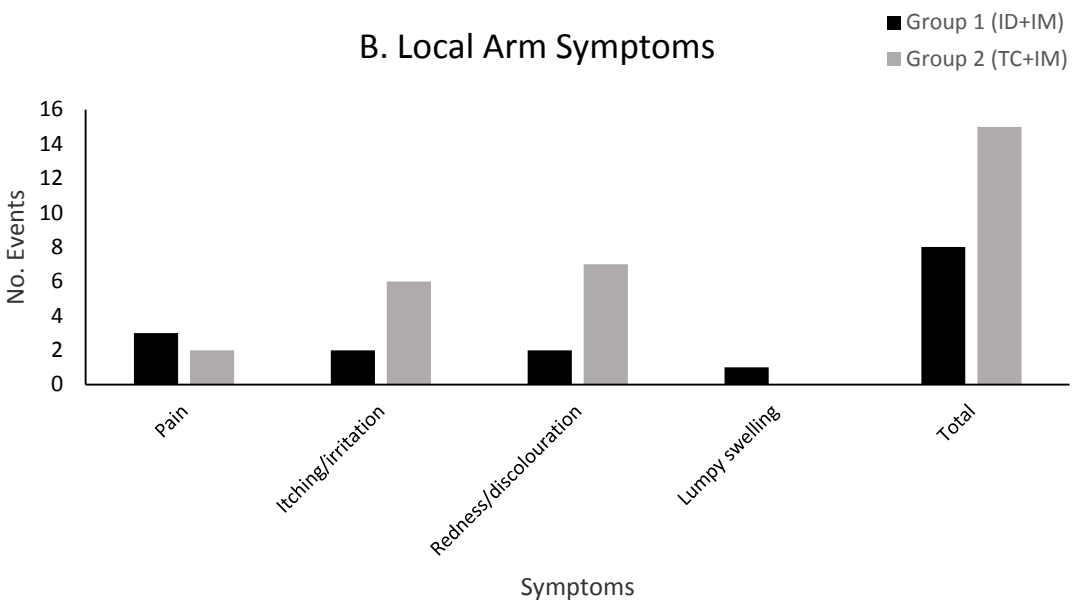
Table 3.5: Summary of clinical safety data (solicited events) from participant diary cards. Statistical significance was set at p=<0.05. NB: For local arm reactions, there are no recordings for the EP+IM group as this group did not receive vaccination to any arm. For local leg reactions the ID+IM and TC+IM groups received intramuscular vaccination to each leg, whereas the EP+IM group received IM vaccination followed by EP at the same site to each leg.

Safety: Adverse events	Group 1 ID+IM	Group 2 TC+IM	Group 3 EP+IM	Total
Definitely related to vaccine	0	0	1	1 (2%)
Possibly related	6	3	0	9 (15%)
Probably related	0	4	0	4 (7%)
Unlikely related to vaccine	20	14	12	46 (76%)
Total	**26**	**21**	**13**	**60 (100)**

Although no statistical significance was found between the groups, there were a greater number of local arm symptoms reported in the TC+IM group compared to the ID+IM group (Figure 3.6B). Of note from 7/11 participants reporting redness/discolouration in the TC+IM group, 5 of these had hypo or hyperpigmentation changes over the transcutaneous vaccine site, most likely a post-inflammatory phenomenon related to the procedure.

With respect to local leg symptoms, a greater number of participants reported pain in the EP+IM group, a reflection of the EP procedure causing local events compared with the other 2 groups who only received IM injection to the thigh (without EP) (Figure 3.6C).





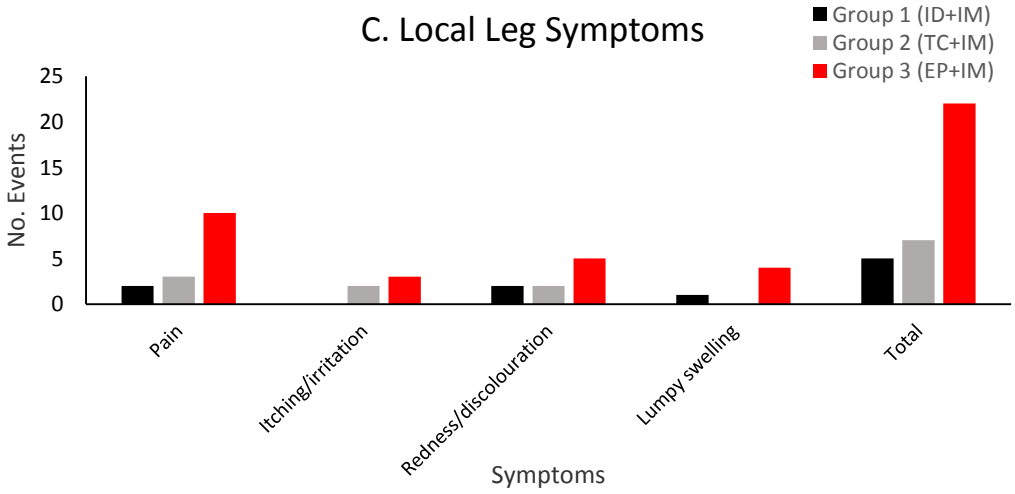
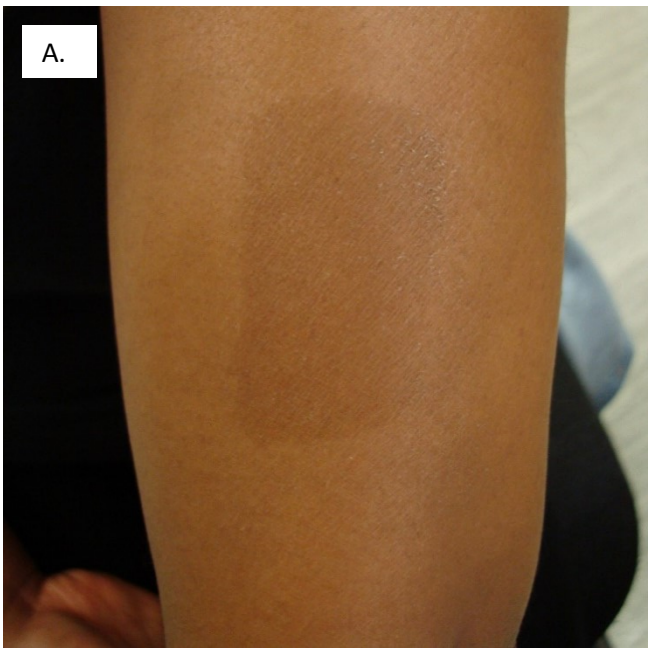


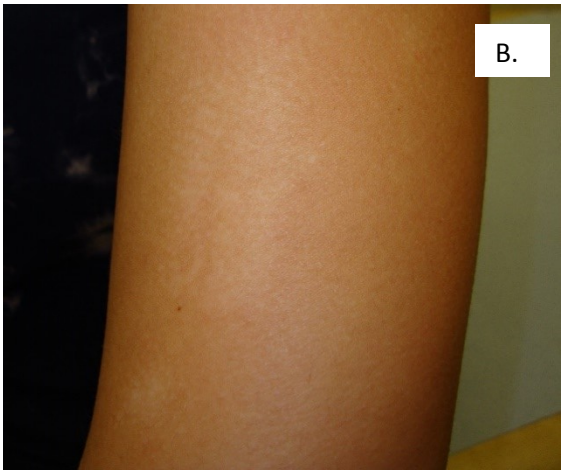
Figure 3.6: Systemic and local reactogenicity events from participant diary cards A: General symptoms documented by participants on their diary cards filled out from D0 to D7 post vaccination. Cumulative results shown from all 3 diary cards where y axis is the number of events and x axis reflect symptoms. B: Local arm symptoms, ID+IM and TC+IM groups only as EP+IM did not receive vaccination to any arm. C: Local leg symptoms documented from all 3 groups where ID+IM and TC+IM groups received IM injection to upper thigh and EP+IM received IM injection followed by EP using the integrated device.

In this study 2/5 participants developed hyperpigmentation. The first participant (C033) was of Black African origin (Nigerian) and developed moderate hyperpigmentation at week 14 (2 weeks after the

final vaccine), which had resolved by week 20. The other participant (C034) was of Indian origin and developed mild hyperpigmentation at week 5 (1 week after the second vaccine) which had resolved by week 12 (the time of the final vaccine) and did not recur.

3 participants developed hypopigmentation in this trial. Participant C052 (Caucasian origin) had mild hypopigmentation 1 week following the second vaccine (week 5) which continued for the reminder of the trial without worsening and with resolution at the final follow up at week 20. Participant C031 was of mixed Black/White origin, developed hypopigmentation over the TC vaccine site at week 14, which lasted well beyond final follow up and did not resolve until 3 months following the end of the trial. Finally, participant C045 declined the final vaccination at week 12 due to ongoing moderate hypopigmentation following the second vaccination. She also developed satellite hypopigmented lesions away from the vaccine site prompting GP referral and an underlying diagnosis of vitiligo likely triggered by cutaneous trauma and inflammation. Her skin changes resolved 6 months following trial completion without treatment for vitiligo. Although in all participants the skin changes resolved, as mentioned above 1 participant in this group declined the final vaccine at week 12 due to ongoing hypopigmentation (Figure 3.7).





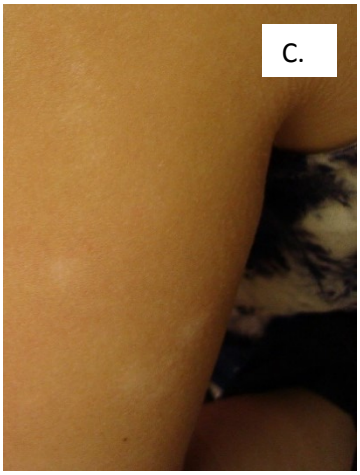


Figure 3.7: A: Example of hyperpigmentation on left upper arm in a trial participant in the TC+IM group. This participant was of Nigerian origin. The hyperpigmentation shown here occurred at week 14, 2 weeks after the final vaccine and had resolved by the final follow up visit at week 20. B: Example of hypopigmentation on left upper arm in a trial participant in the TC+IM group. This participant was of Russian origin and the hypopigmentation occurred at week 5, 1 week after the second vaccine and persisted resulting in the participant declining to receive the final vaccine. C: Example of satellite lesions over posterior upper arm (away from TC vaccine site) in the same participant from figure B, indicating an underlying diagnosis of vitiligo.

3.2.4 Tolerability and acceptability data (EP+IM group only)

Participants in this group were asked to fill out pain questionnaires following each procedure and also an acceptability questionnaire. The visual analog pain score used ranged from 0 (no pain) to 10 (worst ever pain) and participants were asked to rate their pain immediately after EP and 30 minutes later. The maximal response for each leg was documented.

The EP tolerability data shows the peak pain score is immediately after EP with the majority of participants (70%) rating the pain as moderate (4-7/10), with pain improving after 30 minutes (Figure 3.8). The mean pain score immediately after EP was 5.9, and 30 minutes later 2.4 showing a significant decrease in mean pain score (p=<0.0001 Wilcoxon matched-pair t test). In addition, over 90% of participants indicated EP was acceptable if it protected people from a serious disease for which no vaccine was currently available (Figure 3.9).

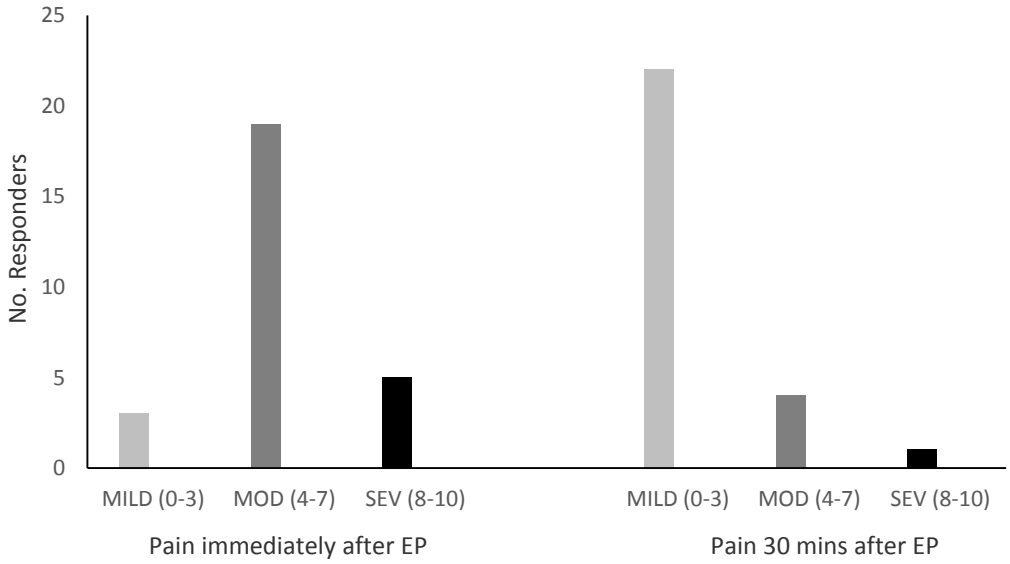
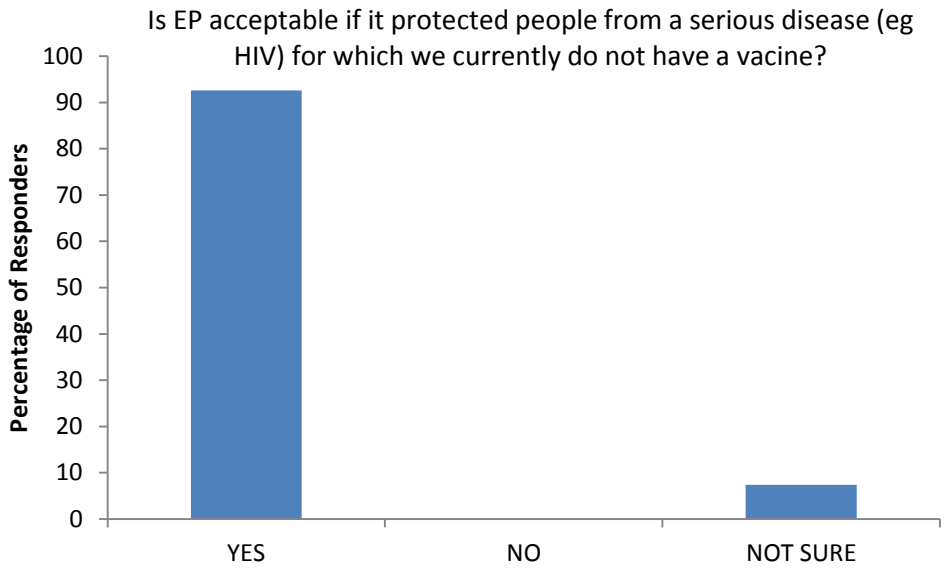


Figure 3.8. Tolerability questionnaire for EP. Cumulative responses from pain questionnaire filled out by participants in this group after every vaccine procedure. Participants were asked to rate their pain out of 10 (1-3=mild; 4-7=moderate; 8-10=severe). Responses shown are cumulative and the maximum response is taken for both legs to give one final response.



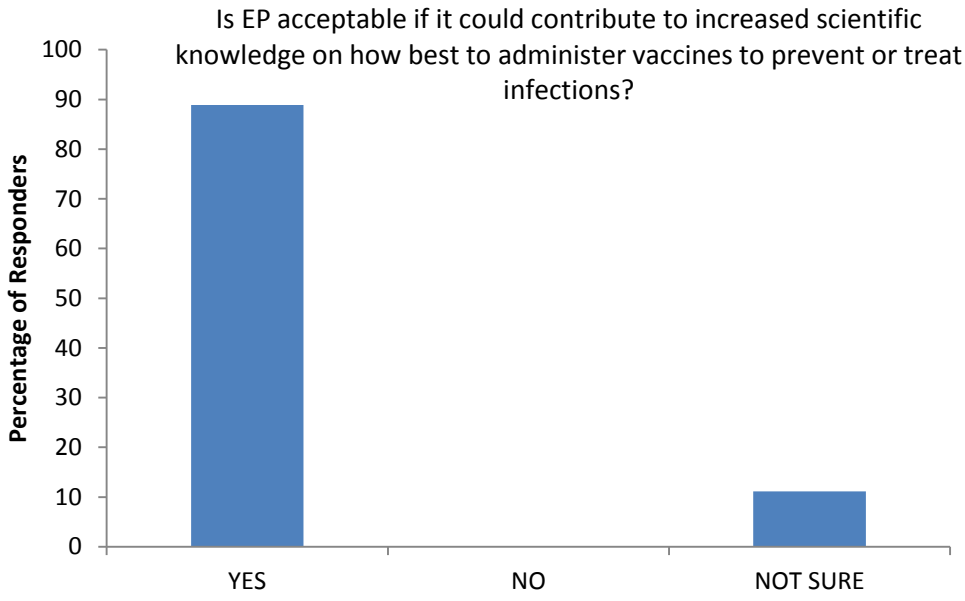


Figure 3.9: Acceptability questionnaire for EP. Question 1 where y axis is percentage of responders voting yes, no or not sure. Question 2 where y axis is percentage of responders voting yes, no or not sure.

3.3 DISCUSSION

In this chapter I aimed to determine the safety of the GTU®MultiHIV B clade DNA vaccine using 3 routes of immunisation in HIV uninfected volunteers participating in the CUTHIVAC 001 randomised phase I clinical trial.

This was my first experience in the active recruitment of volunteers to a clinical trial and recruitment was lengthy and challenging. 420 people showed an interest in the trial yet this translated to less than 60 people coming forward to initial screening. I screened almost double the number of volunteers (59) in order to enrol 30 participants (screening:enrolment ratio 1.9:1), and other HIV-1 vaccine trials have yielded similar challenges in terms of the number of participants screened (1.8:1 in a similar HIV-1 phase I vaccine trial using electroporation) [182].

The most successful recruitment strategy was social media which generated both the greatest number of people enquiring and 30% of participants actually enrolled. The use of facebook, twitter and other digital platforms has expanded in all aspects of day to day life, and should not be underestimated. Previous data has suggested social media could be a valuable tool both for clinical trial recruitment and dissemination of health information if conducted in a stringent manner taking into account ethical and confidentiality concerns [210]. Cost effectiveness is also a factor to consider in recruitment, and our online platforms came in at a total spend of under £100 for the recruitment process (excluding the cost of an individual's time for updating online platforms, estimated at <1hr a day.) The Metro adverts, the second most successful recruitment source, were priced at £2900 per advert (total for 2 adverts = £5800).

In this trial the majority of participants (38%) indicated their main motivation for volunteering was the financial incentive for participating. Participants were paid a total of £1400 pounds for attending all visits (£200 for each vaccine visit and £100 for each safety visit). The payment of volunteers particularly in early phase I trials is not a new concept, but is still weighted with controversy. Outside of clinical trials financial incentives have been attached to several health interventions including aiming to increase exercise in cardiac patients [211], and smoking cessation in pregnant women [212]. In the context of clinical trials, care must be taken to ensure an informed consent process is in place within a robust ethical framework, particularly in relation to phase I trials involving healthy volunteers.

The GTU®MultiHIV B clade DNA vaccine had been previously investigated in both HIV uninfected and infected volunteers. Robust safety data was already available from the FIT-06 trial in HIV positive volunteers in South Africa who were not on ART [135]. In CUTHIVAC 001 there were no serious

adverse events in this trial, and the majority of adverse events (76%) were deemed unrelated to vaccination as evaluated by the trial physician (me) and a safety endpoint committee review. This is similar to FIT-06 where 83% of adverse events were assessed as 'unlikely' related to the investigational product. Further direct comparisons with this trial are limited due to the different populations enrolled in the trial and routes of vaccination.

Although there were no significant differences between groups in local arm reactogenicity, it is important to take into consideration the greater number of local arm reactions in the TC+IM group compared to ID+IM (15 and 8 respectively). The transcutaneous route of vaccination has not been used to the best of my knowledge in a previous HIV vaccine trial, nor with DNA. Data from a clinical trial investigating a combined influenza and tetanus vaccine (Tetragrip), using TC vaccination alone in comparison to IM vaccination reported although there were moderate local reactions in TC vaccinated individuals compared to IM, no significant increases in localized adverse events were reported between groups with no reported pigmentation skin changes [191]. It is important to note in that trial in contrast to CUTHIVAC 001, all participants were of skin types II or III (white skin) with no darker skin types included.

5 participants in the TC+IM group in CUTHIVAC 001 developed post-inflammatory hypo or hyperpigmentation over the TC vaccine site, which may have been as a result of the CSSS procedure, the vaccine, or a combination of the two. Hyperpigmentation may occur secondary to trauma and inflammation of the skin, with darker skin tones likely to be more prone to this condition [213]. The mechanism is thought to be secondary to destruction of the basal skin layer and a cutaneous inflammatory response resulting in the release of melanin [214]. Previous studies using the CSSS technique have excluded darker skin tones, but local inflammatory reactions to date have been mild or moderate, therefore for this trial there were no such exclusions. Post-inflammatory hypopigmentation is an acquired loss of skin pigmentation also following cutaneous inflammation. There is limited information on the mechanism in the literature, but some data suggests 'weak' melanocytes with a high predisposition to damage may be a contributing factor [215].

Although all the skin changes resolved, the resulting post-inflammatory changes led to 1 participant declining the final vaccine and may be an important consideration in future trials using this route of vaccination.

Similarly with local leg reactogenicity there were a greater number of participants in the EP+IM group reporting pain compared to other groups. Although this did not reach statistical significance the first participant randomised to EP+IM declined to continue after the first EP procedure. This was the first participant we electroporated in the context of a clinical trial. We also experienced technical

issues with the device during the procedure, all of which may have elevated anxiety in the participant contributing to her final decision of declining to receive further EP. Reassuringly, no other participants declined the procedure following the first EP randomisation, and this is in line with other clinical studies using EP which have demonstrated overall tolerability and acceptability of the procedure [216], [217]. This experience however did lead to modification of the protocol for the CUTHIVTHER vaccine trial using EP, and is discussed in a later chapter.

In terms of limitations, with small participant numbers in each group although safety data is encouraging, greater numbers may be needed in future clinical trials. In addition, there may have been bias introduced into the EP tolerability and acceptability questionnaires as perhaps participants may have felt obliged to downgrade pain scores particularly if filling out forms in front of the clinical team during visits. Finally in terms of the skin changes seen in the TC group, further information may have been useful to collect such as skin biopsies or changes in inflammatory markers but unfortunately this was beyond the remit of this trial or my research project.

In summary, although there were no clinician-led decisions to discontinue vaccination, there were 2 participant decisions to discontinue which need to be taken into consideration if implementation of these vaccination routes is to be considered on a larger scale. Despite these limtiations however, overall the vaccine was safe and tolerable across all 3 groups with no serious adverse events.

Summary of trial data for CUTHIVAC 001

- The GTU®MultiHIV B clade DNA vaccine was overall safe across all 3 groups with no adverse events recorded over Grade 2
- There were 2 participant decisions to discontinue vaccination. TC+IM: ongoing moderate hypopigmentation at TC vaccine site; EP+IM: pain associated with electroporation
- Although the first participant in the EP+IM group declined to continue due to pain, overall participants documented EP was an acceptable route of vaccine delivery
- There were no serious adverse events in this trial

Chapter 4
Results: Immunogenicity Data CUTHIVAC 001

SECTION A: IFN-ɣ T CELL ELISPOTS

4.1 INTRODUCTION

Hypothesis

3. DNA vaccination induces HIV-specific T cell immunity in **HIV negative** volunteers, and is influenced by the route of vaccine administration
4. DNA vaccination induces HIV-specific immunity that confers protection in a viral challenge *in vitro* system in **HIV negative** volunteers

Aims/Objectives

3. To characterise T cell responses in **HIV negative** volunteers immunised with a DNA vaccine, and to determine whether T cell responses are influenced by the route of vaccine administration
4. To determine functionality of HIV-specific T-cell responses in a novel *in vitro* viral inhibition assay amongst **HIV negative** volunteers immunised with an HIV DNA vaccine

In this section, I discuss the immunogenicity results for CUTHIVAC 001, focusing on the first half of my hypothesis and aims in HIV negative volunteers. I conducted all the IFN-ɣ ELISPOT assays and the VIA myself. The intracellular cytokine staining (ICS) data is presented as supporting work as I did not conduct the assay myself, but did analyse the results which are included here to show an additional evaluation of the quality of the immune response.

IFN-ɣ production by peptide stimulation of T cells has been extensively used to quantify and characterise cellular immune responses in HIV-1 vaccine trials, as well as in other diseases including TB and malaria [218], [219]. The IFN-ɣ T cell ELISpot assay has several advantages over other assays including reproducibility, cost effectiveness, and the ability to rapidly screen a wide variety of

antigens [200]. However, in interpreting my ELISpot results it remains important to remember although IFN-ɣ is conventionally used in vaccine trials, it represents one cytokine response only and therefore may not be an adequate marker of immune function or represent the quality or function of the immune response. The limitations of this assay are covered in the discussion section of this chapter and compared to data from ICS and the VIA.

ICS of stimulated PBMCs followed by flow cytometry is a well-established method for detecting expressed cytokines, and has the added advantage over the T cell ELISpot assay of simultaneous expression of numerous cytokines that can characterise T cell phenotypes [220]. In HIV vaccine trials this may be particularly important as the correlates of immune function are still not fully understood. Using ICS can provide both a quantitative phenotypic and functional approach to further understand T cell vaccine responses, with recent publications aimed at standardizing ICS techniques for consistency of results in clinical trials [221].

All the immunogenicity analyses presented below are based on an intention to treat (ITT) analysis where samples from all time points from all participants are included. A reminder of the vaccination schedule in this trial is shown (Table 4.1).

Table 4.1: Vaccine schedule for CUTHIVAC 001. Vaccination time points, safety visits and final visit shown. The primary end point is highlighted in red.

Visit number	1	2	3	4	5	6	7	8	9	10	11
Nature of visit	Screen	Dosing visit	Safety visit	Safety visit	Dosing visit	Safety visit	Safety visit	Dosing visit	Safety visit	Safety visit	Follow-up visit
Week	-6	0	0+1day	1	4	4+1day	5	12	12+1day	14	20
										Primary end point	

Overview of methods and time points tested

For a detailed overview of the immunogenicity sampling schedule please refer to Table 2.3 in Chapter 2: methods. In brief, bloods samples were collected from participants at the specified time points with PBMC processing according to GcLP. Samples were stored in batches at -80˚C until analysis for immunogenicity outputs.

A response was considered positive if the following criteria were met: 1) The positive control was >1000 spot forming units per millions PBMC (SFU/M); 2) The mean of the negative wells was <100 SFU/M; 3) The positive response was >4 x mean background (or greater than 0 if the background was 0) and >55 background subtracted SFU/M. These definitions have been applied to previous HIV-1 vaccine trials [199],[200].

The magnitude of the IFN-ɣ response refers to the number of SFU/M PBMC with background subtracted as stated above. Breadth corresponds to the number of responses to the different peptide pools. Finally the duration of the IFN-ɣ response denotes the presence of a positive response at the final follow up appointment at 20 weeks (6 weeks after the primary end point).

4.2 RESULTS: IFN-ɣ T CELL ELISPOTS

4.2.1 Electroporation preferentially expands IFN-ɣ responses at the primary end point

As stated previously the primary end point in this trial was at week 14, 2 weeks after the third and final vaccination. At the primary end point, a positive IFN-ɣ ELISpot response to any peptide was seen in 1/9 participants in the ID+IM group (11%), 0/11 in the TC+IM group and 9/10 (90%) in the EP+IM group (Table 4.2).

The magnitude of the IFN-ɣ response was greatest in the EP+IM group across all peptide pools (Table 4.2). In addition, there were a greater number of responders in the EP+IM group to each peptide pool at the primary end point with 5/10 responders to *Rev*, 3/10 to *Tat*, 9/10 to *Nef*, 9/10 to *Gag* and 6/10 to *CTL* (Figure 4.1). The only responder in the ID+IM group was to *Gag.* Of note, it appears there are 2 further responders in the ID+IM group to the *Nef* peptide pool, however although these 2 participants may have met one of the 'positive responder' criteria (>55 background subtracted SFU/M), the second criteria was not met (positive response >4 x mean background) and therefore these participants are not included as positive responders at the primary end point.

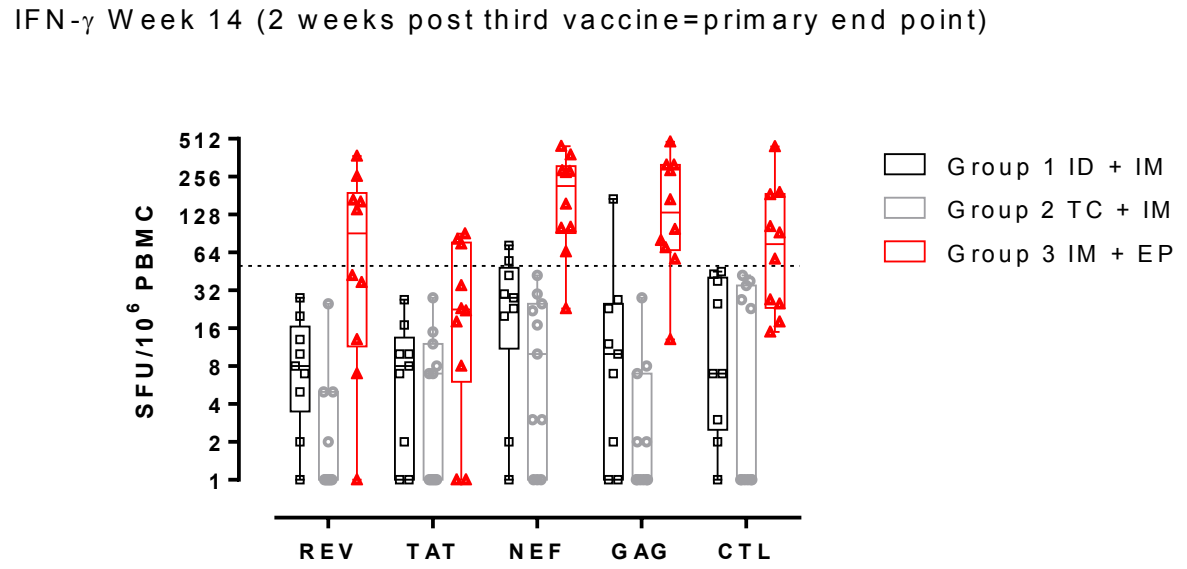


Figure 4.1: IFN-ɣ response at the primary end point shown by each peptide pool and group. All units are in SFU/M with background subtracted. The dotted line at 55 SFU/M PBMC denotes a positive response, although participants also had to meet other criteria for a positive response. The scale shown is $\log^2$.

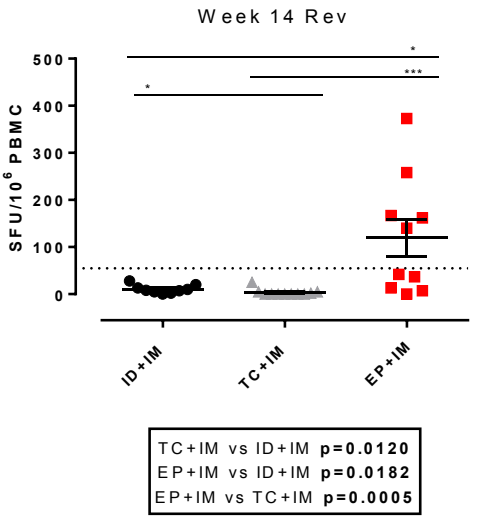
Table 4.2: IFN-ɣ responses at week 14 shown by group and per peptide with mean, median and p values shown. Statistical significance is set at p=<0.05.

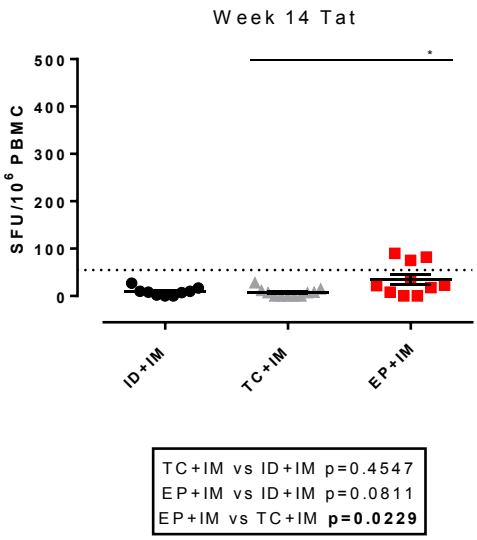
	Group 1 ID+IM	Group 2 TC+IM	Group 3 EP+IM	**Total (%)**
IFN-ɣ response at primary end point (n/total)	1/9 (11%)	0/11 (0%)	9/10 (90%)	10/30 (33%)
Rev (SFU/M)				
Mean (SEM)	10 (3)	3 (2)	120 (39)	TC+IM vs ID+IM **p=0.0120**
Median	8	0	91	EP+IM vs ID+IM **p=0.0182**
25-75 percentile	4-16	0-5	12-190	EP+IM vs TC+IM **p=0.0005**
Tat (SFU/M)				
Mean (SEM)	9 (3)	7 (3)	35 (10)	TC+IM vs ID+IM p=0.4547
Median	8	7	23	EP+IM vs ID+IM p=0.0811
25-75 percentile	1-13	0-12	6-77	EP+IM vs TC+IM **p=0.0229**
Nef (SFU/M)				
Mean (SEM)	30 (8)	14 (4)	212 (45)	TC+IM vs ID+IM p=0.1455
Median	28	10	216	EP+IM vs ID+IM **p=0.0007**
25-75 percentile	11-49	0-25	91-311	EP+IM vs TC+IM **p=<0.0001**
Gag (SFU/M)				
Mean (SEM)	28 (18)	4 (3)	189 (49)	TC+IM vs ID+IM p=0.0737
Median	10	0	133	EP+IM vs ID+IM **p=0.0014**
25-75 percentile	1-25	0-7	67-317	EP+IM vs TC+IM **p=<0.0001**
CTL (SFU/M)				
Mean (SEM)	19 (6)	16 (5)	116 (42)	TC+IM vs ID+IM p=0.3518
Median	7	7	75	EP+IM vs ID+IM **p=0.0109**
25-75 percentile	3-41	0-35	23-187	EP+IM vs TC+IM **p=0.0063**

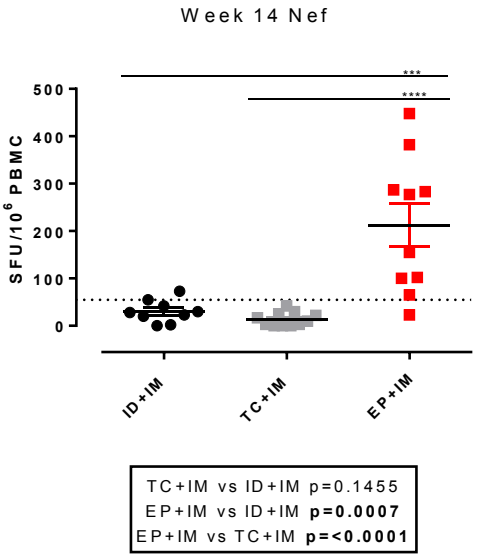
To assess whether there was a difference in the magnitude of the IFN-ɣ response between the groups for each peptide an ordinary one-way ANOVA comparing all 3 groups was initially conducted, which showed a statistical significance between the groups for each peptide. An unpaired t-test (non-parametric Mann-Whitney test) between groups for each peptide was then conducted (Figure 4.2).

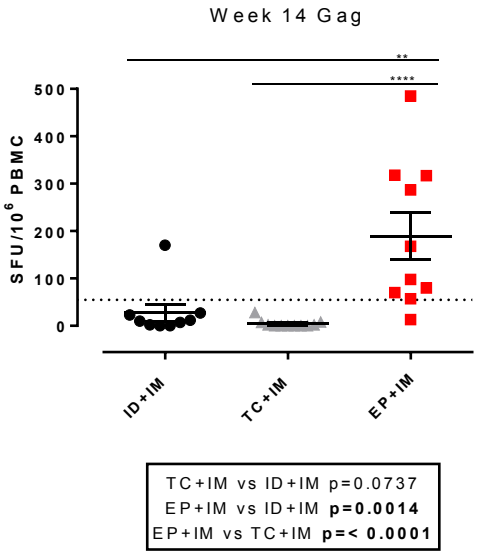
The results for *Rev* showed a statistically significant difference between all three groups, with the greatest significance between EP+IM and TC+IM (p=0.0005). With respect to the *Tat* peptide pool, the only significance was between EP+IM and TC+IM groups (p=0.0229). For the remaining 3 peptide pools (*Nef, Gag, CTL*), there was statistical significance between EP+IM and the other 2 groups, but no significant difference between TC+IM and ID+IM.

The difference in magnitude between groups is also apparent by examining the stacked peptide response at week 14 (Figure 4.3). Each bar represents an individual participant with all responses stacked to give an overall pooled magnitude, with the EP+IM group displaying the greatest magnitude of pooled peptide responses compared to the other groups.









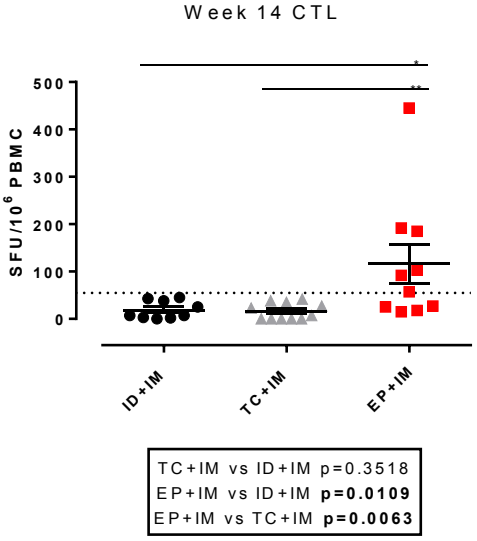


Figure 4.2: Group comparisons for each peptide pool at week 14. All units are in SFU/M PBMC with background subtracted. Statistical test performed: Unpaired t test (non-parametric Mann-Whitney test) with significance set at p=<0.05.

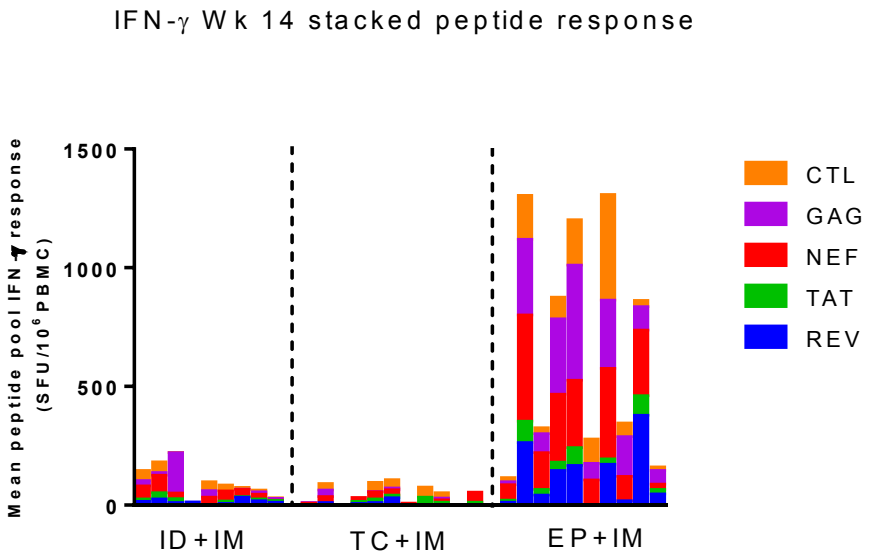


Figure 4.3: Stacked peptide response where each bar represents a participant showing the mean pooled response for each participant.

4.2.2 Individual group primary end point analysis

ID+IM (comparison of week 0 to week 14)

From the stacked peptide pool response in the ID+IM group comparing week 0 to week 14, there appears to be little difference post vaccination (Figure 4.4). However, a paired t test (non-parametric Wilcoxon test) of all peptides showed a significant difference comparing week 0 to week 14 for all peptides pooled together (p=0.0071). Once this statistical test is run for each individual peptide however, it is only the *Nef* peptide pool which shows statistical significance (p=0.0391) (Figure 4.5).

TC+IM (comparison of week 0 to week 14)

In this group there were no significant differences between week 0 and week 14 for any peptide (Figure 4.6)

EP+IM (comparison of week 0 to week 14)

In this group, there was a highly statistically significant difference comparing all peptides at week 0 and week 14 (p=<0.0001) and when this was broken down into each individual peptide pool all showed significant differences with the greatest difference in the *Nef* and *Gag* peptide pools (p=0.0020 in both) (Figure 4.7). This correlates with the greatest number of responders in the *Nef* and *Gag* peptide pools compared to the other peptide pools at 8/10 and 9/10 positive responders respectively (Figure 4.8).

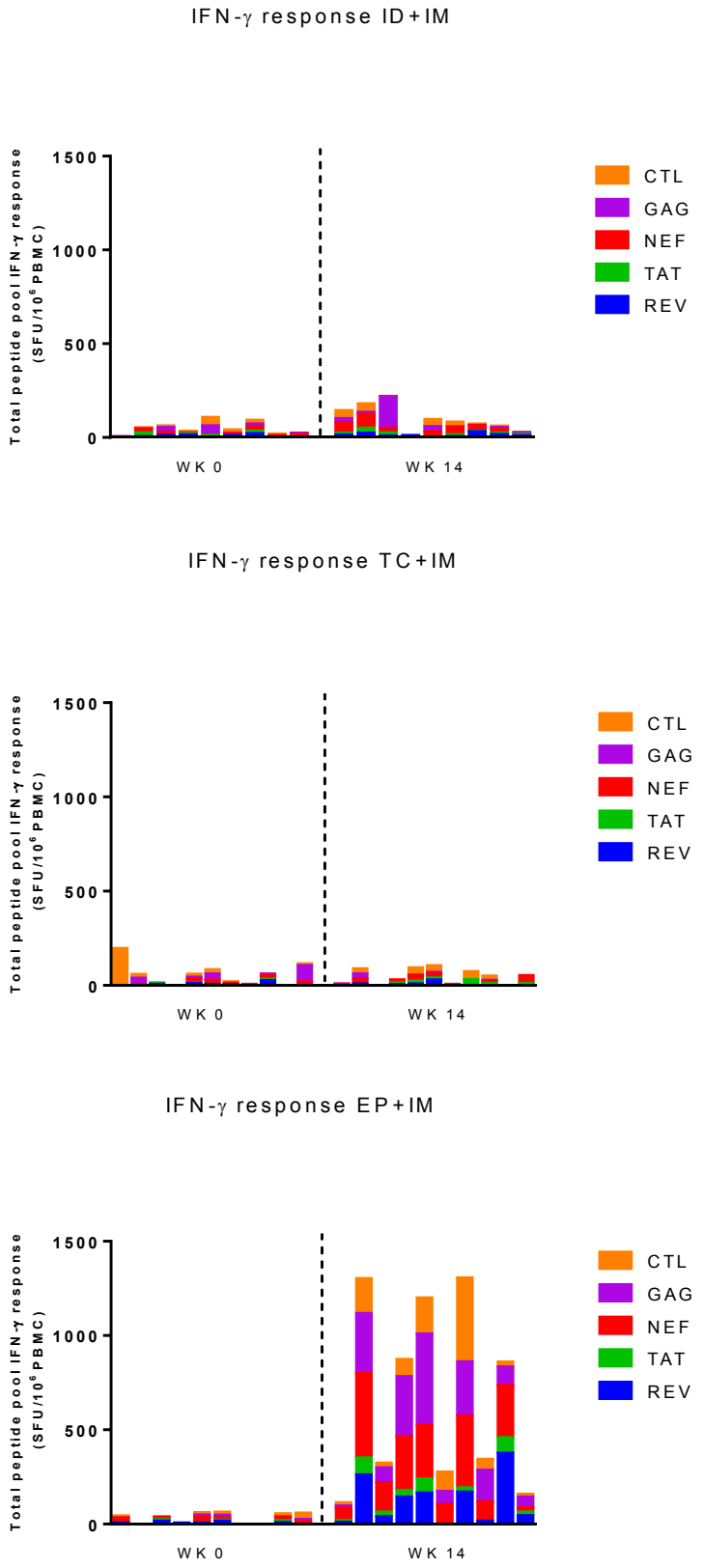


Figure 4.4: Graphs show the stacked IFN-ɣ response with each peptide pool indicated by colour (see key) in each group comparing week 0 to week 14. All responses are presented as SFU/M PBMC with background subtracted.

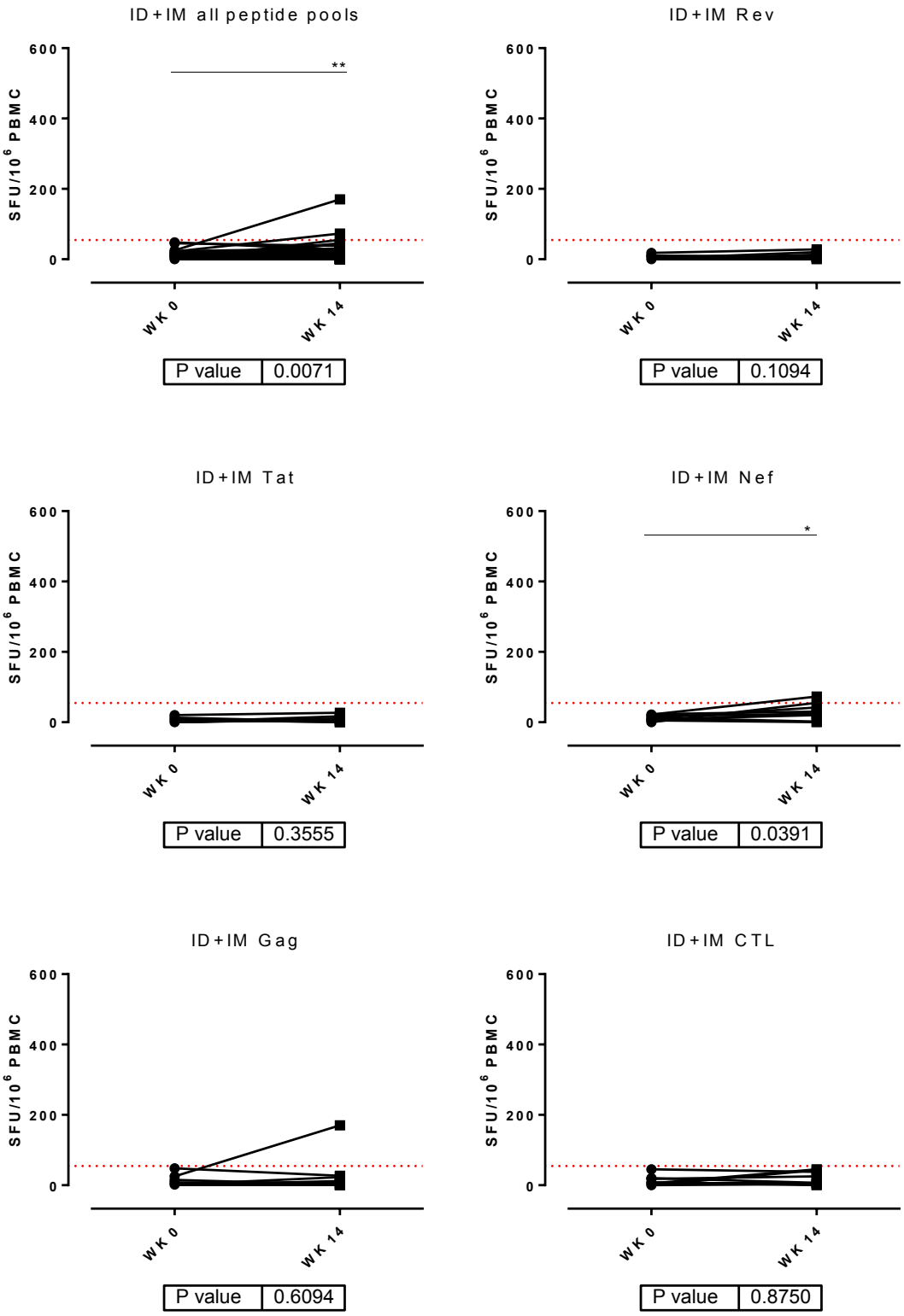


Figure 4.5: ID+IM Before/After graphs showing difference in magnitude of IFN-ɣ response from week 0 to week 14 initially with all peptide pools together (top left graph) and then individually. Statistical significance is set at p=<0.05. Each dot joined together by a line is an individual's IFN-ɣ response at week 0 and 14.

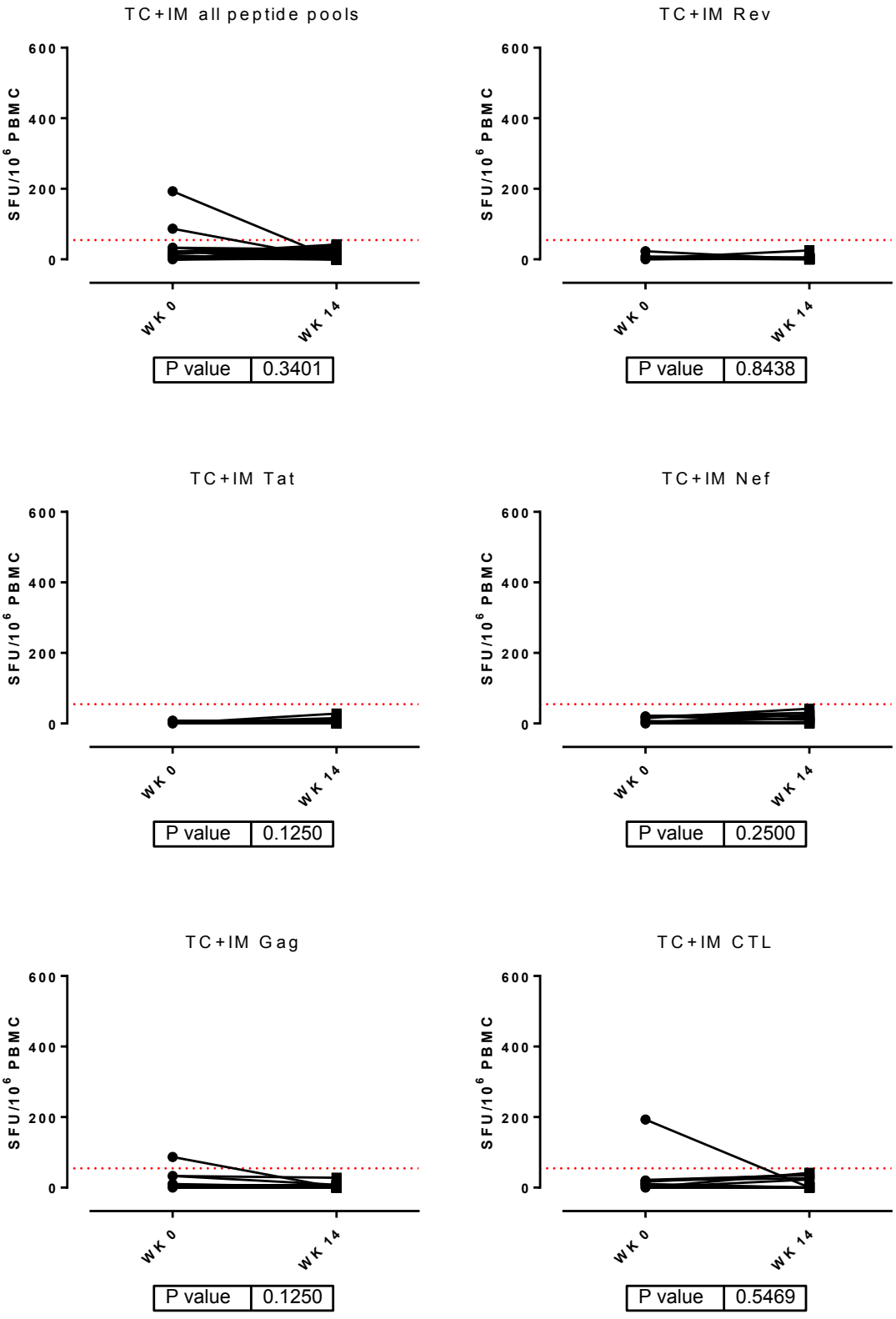


Figure 4.6: TC+IM Before/After graphs showing difference in magnitude of IFN-ɣ response from week 0 to week 14 initially with all peptide pools together (top left graph) and then individually. Statistical significance is set at p=<0.05. Each dot joined together by a line is an individual's IFN-ɣ response at week 0 and 14.

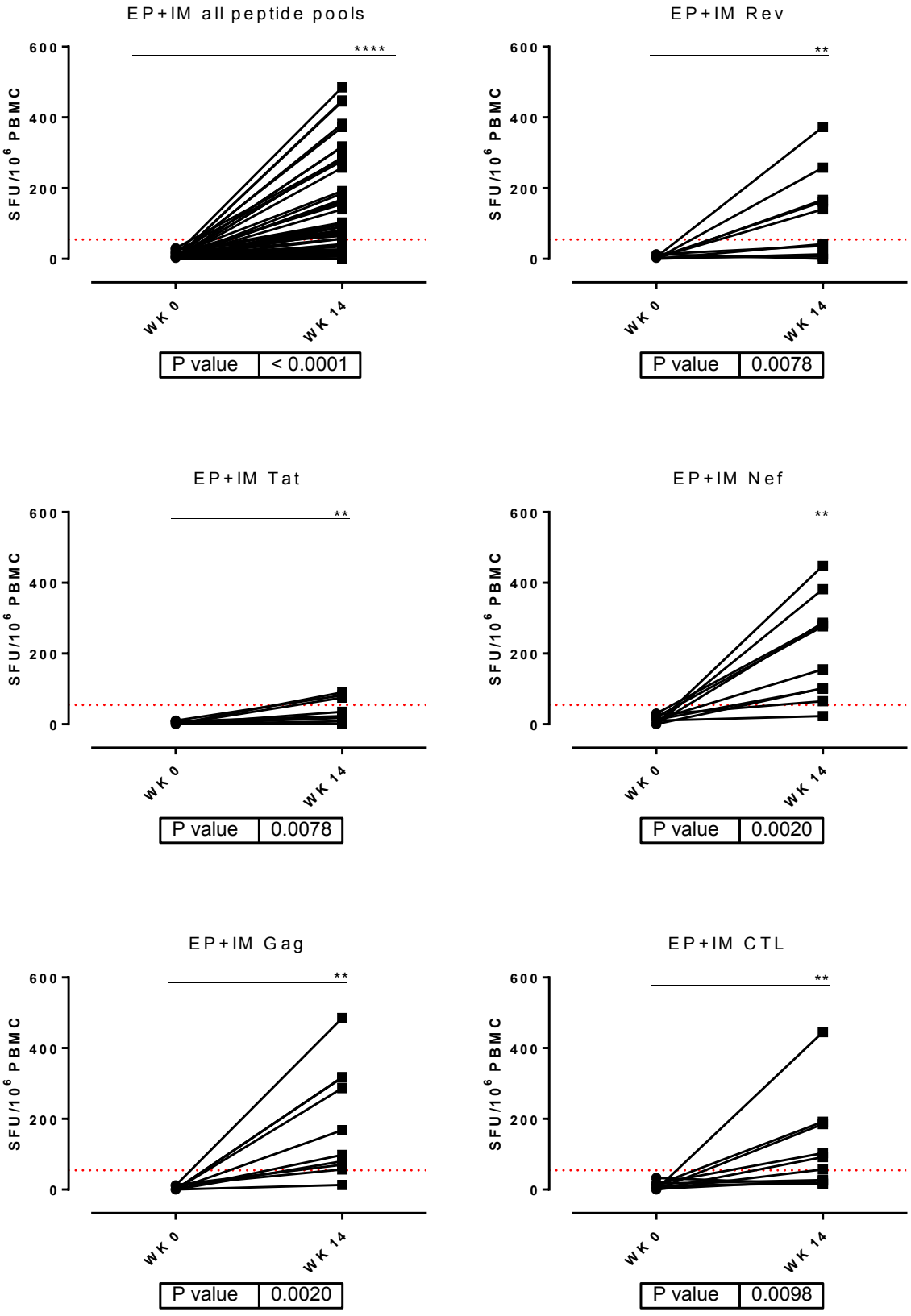


Figure 4.7: EP+IM Before/After graphs showing difference in magnitude of IFN-ɣ response from week 0 to week 14 initially with all peptide pools together (top left graph) and then individually. Statistical significance is set at p=<0.05. Each dot joined together by a line is an individual's IFN-ɣ response at week 0 and 14.

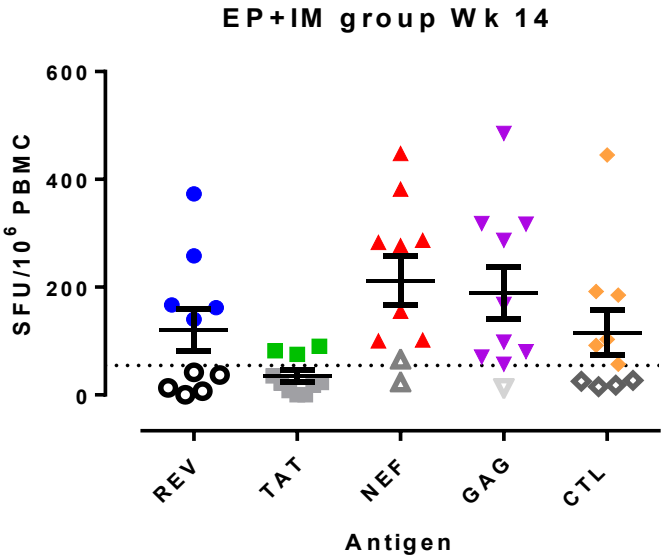


Figure 4.8: Scatter plot showing participants in EP+IM group at week 14. Each coloured dots represents a positive responder with 4/10 responders to *Rev*, 3/10 responders to Tat, 8/10 responders to *Nef*, 9/10 responders to *Gag* and 6/10 responders to *CTL*. The dotted line is set at 55 SFU/M to denote the cut-off for a positive response, however this was not the only criteria set for a positive responder as explained previously, therefore there may appear to be some participants >55 SFU/M who are not included as a positive responder.

4.2.3 IFN-ɣ responses across the interim time points (weeks 1 to 12) are variable with the greatest number of responders at any time point in the EP+IM group

PBMC samples were also analysed using the IFN-ɣ ELISpot assay at interim time points when participants were seen for their safety visits (Table 2.3 Immunogenicity sampling schedule in methods section).

In the ID+IM group, there were a total of 2/9 (22%) participants with a positive response at any time point, including the primary end point. One participant C025 had a positive response to the *Gag* peptide pool at almost all visits excluding week 12 (third vaccine visit) (Table 4.3 & Figure 4.9A), with an additional participant C040 responding to *Gag* at week 4 only.

In the TC+IM group, there were a total of 6/11 (55%) participants with a positive response at any time point, but as stated previously no participants showed a positive response to any peptide pool at the primary end point. C009 had a high background response to *CTL* pre vaccination (week 0), which was present throughout most other time points and therefore this may not represent a 'true' response (Figure 4.9B) and should be disregarded. There were 2 other participants with a positive

response at week 4 to *Gag* (C011 and C031), and 3 different participants responding to *CTL* and *Nef* at week 20.

Finally, in the EP+IM group 9/10 participants had positive responses at any time point, including at the primary end point. Although there were no responses at Week 1 (1 week post first vaccine), 6 participants had a positive response at week 4, 2 at week 5, 7 at week 12 and 8 at week 20. Similar to the other groups, an early response to *Gag* is also noticeable (Figure 4.10).

Table 4.3: Individual responders at each time point with response to peptide pool shown

	ID+IM	TC+IM	EP+IM	TOTAL
Week 0		C009 (CTL)		**1/30**
Week 1	C025 (gag)	C009 (CTL)		**2/30**
Week 4	C025 (gag) C040 (gag)	C009 (gag, CTL) C011 (gag) C031 (gag)	C021 (nef) C030 (gag) C036 (gag) C043 (gag) C049 (nef, gag) C058 (CTL)	**11/30**
Week 5	C025 (gag)	C009 (nef, CTL)	C036 (rev, nef, gag) C049 (gag)	**4/30**
Week 12		C009 (CTL)	C029 (rev, nef, gag, CTL) C030 (nef) C036 (rev, tat, nef, gag, CTL) C043 (nef, CTL) C049 (nef, gag) C053 (rev, nef) C058 (nef)	**8/30**
Week 14	C025 (gag)		C021 (rev, tat, nef, gag, CTL) C029 (nef, gag) C030 (rev, nef, gag, CTL) C036 (rev, tat, nef, gag, CTL) C037 (nef, gag, CTL) C043 (rev, nef, gag, CTL) C049 (nef, gag, CTL) C053 (rev, tat, nef, gag) C058 (gag)	**10/30**
Week 20	C025 (gag)	C034 (CTL) C048 (nef) C050 (tat, nef)	C021 (rev, nef, gag, CTL) C029 (nef) C030 (nef, gag) C036 (rev, nef, gag, CTL) C043 (rev, nef, gag, CTL) C049 (nef, gag, CTl) C053 (rev, tat, nef, gag) C058 (gag)	**12/30**
Total number of **different** responders per group (%)	**2/9 (22%)**	**6/11(55%)**	**9/10 (90%)**	**17/30**

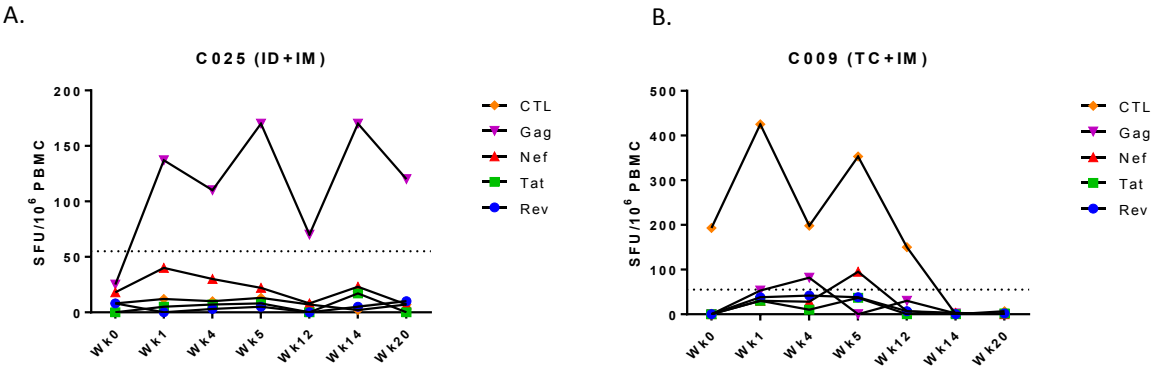


Figure 4.9 A: Gag response shown across time for participant C025 in the ID+IM group. B: CTL response shown across time for C009 in the TC+IM group indicating a high baseline response at week 0.

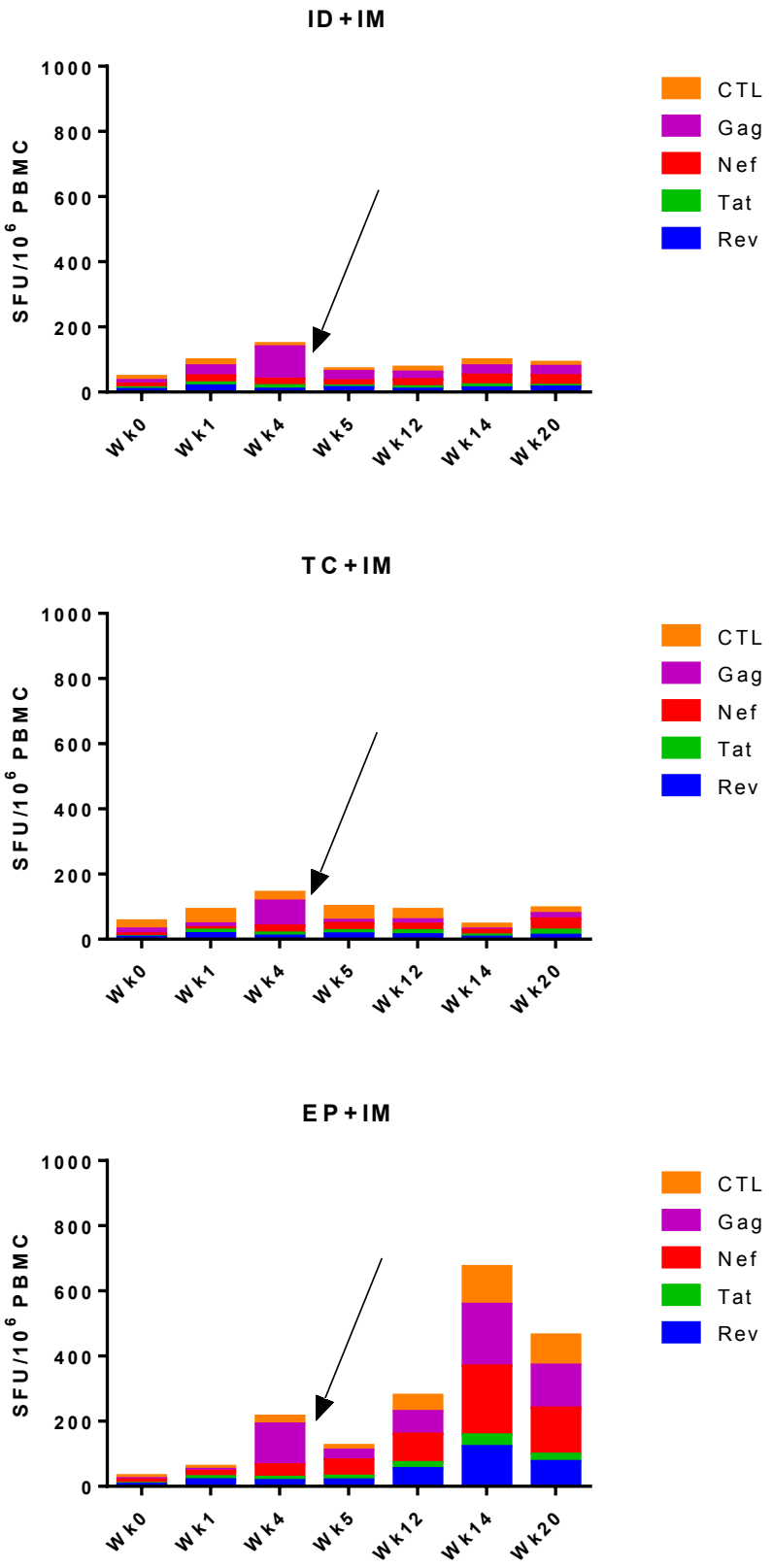


Figure 4.10: Mean pooled peptide response across time shown by group with arrows indicating the presence of an early *Gag* response at week 4 (pre 2nd vaccination). The *Gag* peptide pool is shown in purple.

4.2.4 Electroporation with IM vaccination showed the greatest breadth of the IFN-ɣ response across all time points

There were only 2 participants responding to any peptide across all time points in the ID+IM group. C025 showed a consistent response to *Gag* across almost all weeks (excluding week 0 pre vaccination and week 12). C040 showed an early positive response to *Gag* at week 4 which was then not sustained at later time points (Figure 4.11).

In the TC+IM group, there were 6 responders to any peptide across all time points, with 5 participants responding to one peptide and 1 participant responding to 2 peptides. C009 as mentioned previously had a high background response to *CTL* which was sustained through most weeks (excluding latter time points week 14 and 20). 2 additional participants had an early positive response to *Gag* at week 4 which was not sustained at later time points. At week 20 (final follow up and 8 weeks after final vaccination) 3 additional participants with no responses previously at any time point had positive responses to *CTL* (C034), *Nef* (C048), and both *Tat* and *Nef* (C050).

In the EP+IM group there was a greater breadth of peptide response across most time points beginning at week 4, with 9 responders across all time points (Figure 4.12A). The only participant who did not respond at any time point was C011- this participant did not complete the vaccination schedule and only received the first vaccination (see consort diagram).

The median breadth of peptide response in the EP+IM group peaked at week 14 (4 peptides) as expected at the primary end point. In addition, and looking in more detail at week 14, all participants showed a positive response to at least 1 peptide pool, with a mean breadth of 3.1 peptide pools, and a median of 4 (Figure 4.12B).

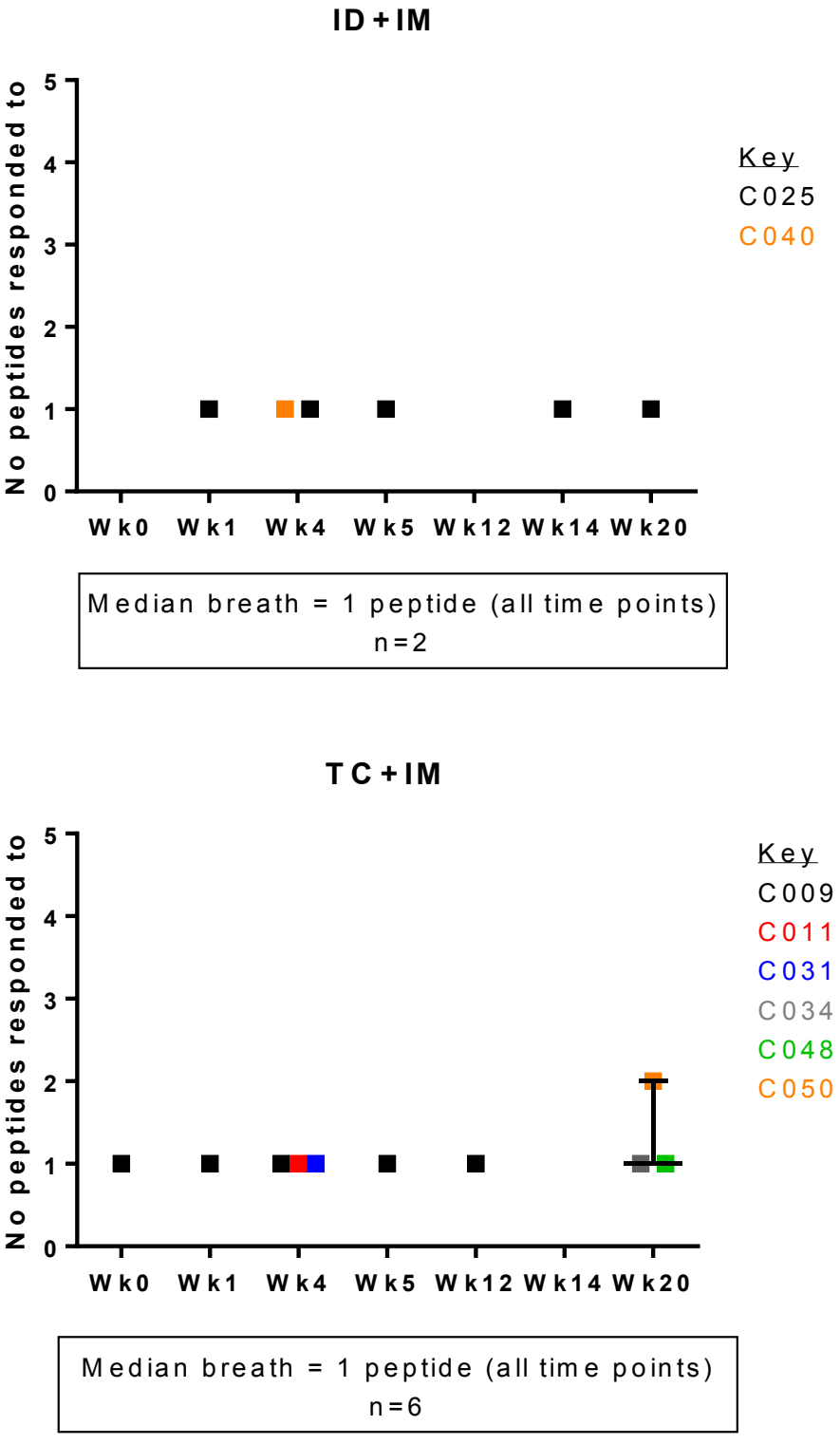
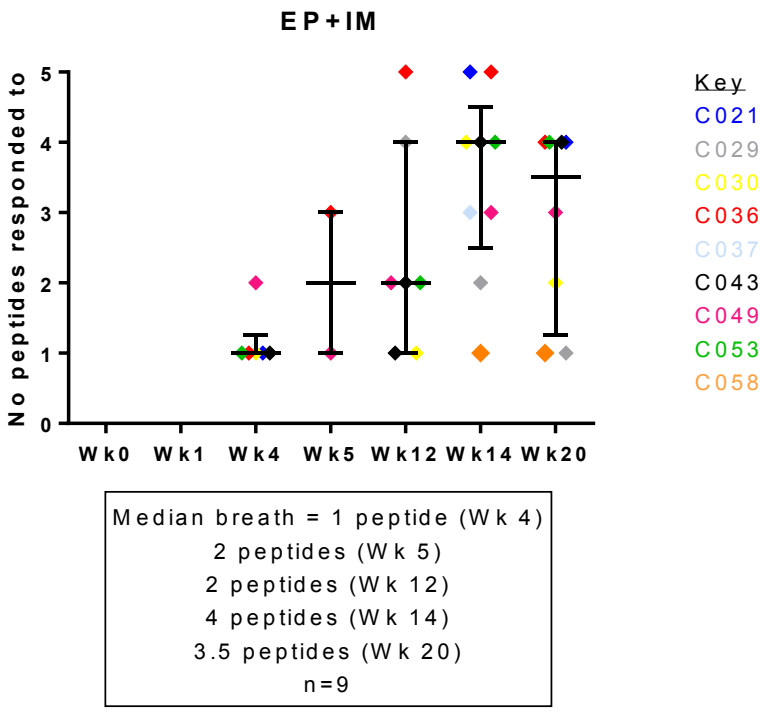


Figure 4.11: Breadth of the response to the peptide groups where breadth is defined as the number of peptide pools a participant responds to (shown on the y axis). The median breadth across all time points is shown here with interquartile range where appropriate for the ID+IM and TC+IM groups. Each participant has been allocated a different colour to show whether the same or different participants are responding at each time point.

A.



B.

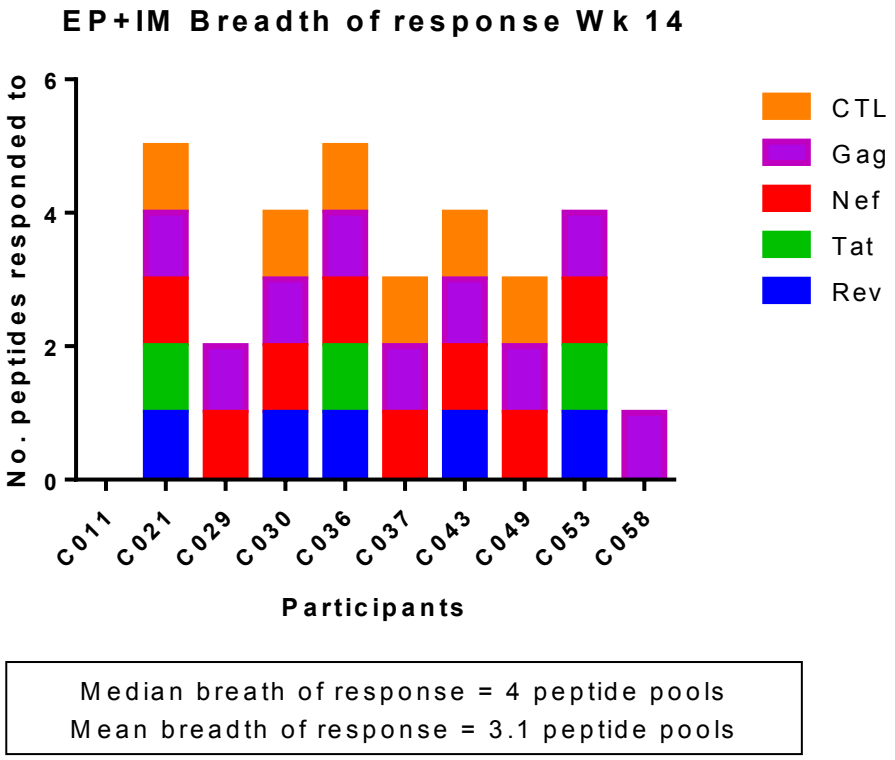


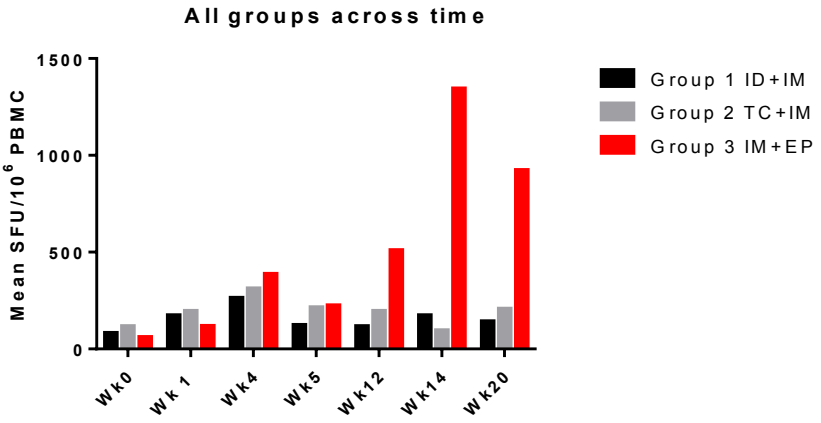
Figure 4.12: A: Breadth of the response to peptide groups in the EP+IM group with median breadth and interquartile range shown. Each participant in this group is allocated a different colour to show whether it is the same participants responding at each time point. B: Week 14 breadth only, broken down by participant to show which peptide each participant has responded to.

4.2.5 The duration of the IFN-ɣ response was greatest in the EP+IM group out to week 20

In this trial the final follow up point was at week 20, 8 weeks following the third vaccination. At week 20, the mean SFU/M PBMC is greatest in the EP+IM group, with 4/10 responders to *Rev*, 1/10 responders to *Tat*, 7/10 responders to *Nef*, 7/10 responders to *Gag,* and 4/10 responders to *CTL* (Figure 4.13 A and B).

To determine statistical significance between week 0 and week 20, a paired t test (Wilcoxon matched-pairs signed rank test) was run, which indicated a significant response in all 3 groups with the greatest statistical significance in the EP+IM group (Figure 4.14).

A.



B.

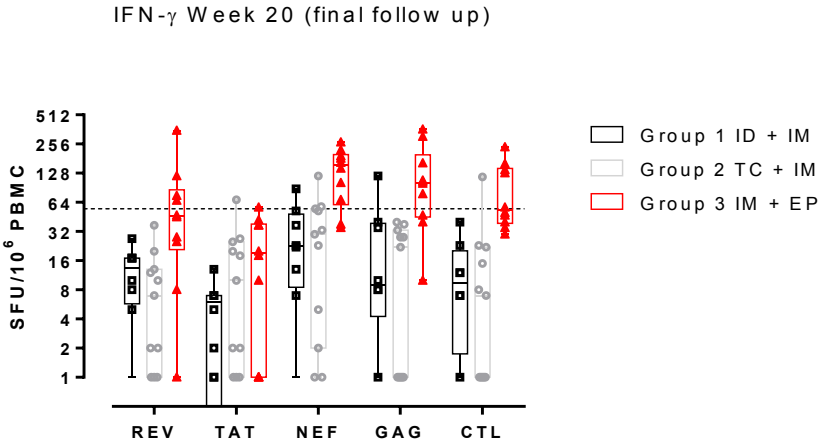


Figure 4.13 A: Mean SFU/M PBMC shown across time by group. B: IFN-ɣ response at week 20 shown by group and peptide pool. The dotted line indicates the cut off of >55 SFU/M PBMC and the scale is a $\log^2$ in order to show all points clearly

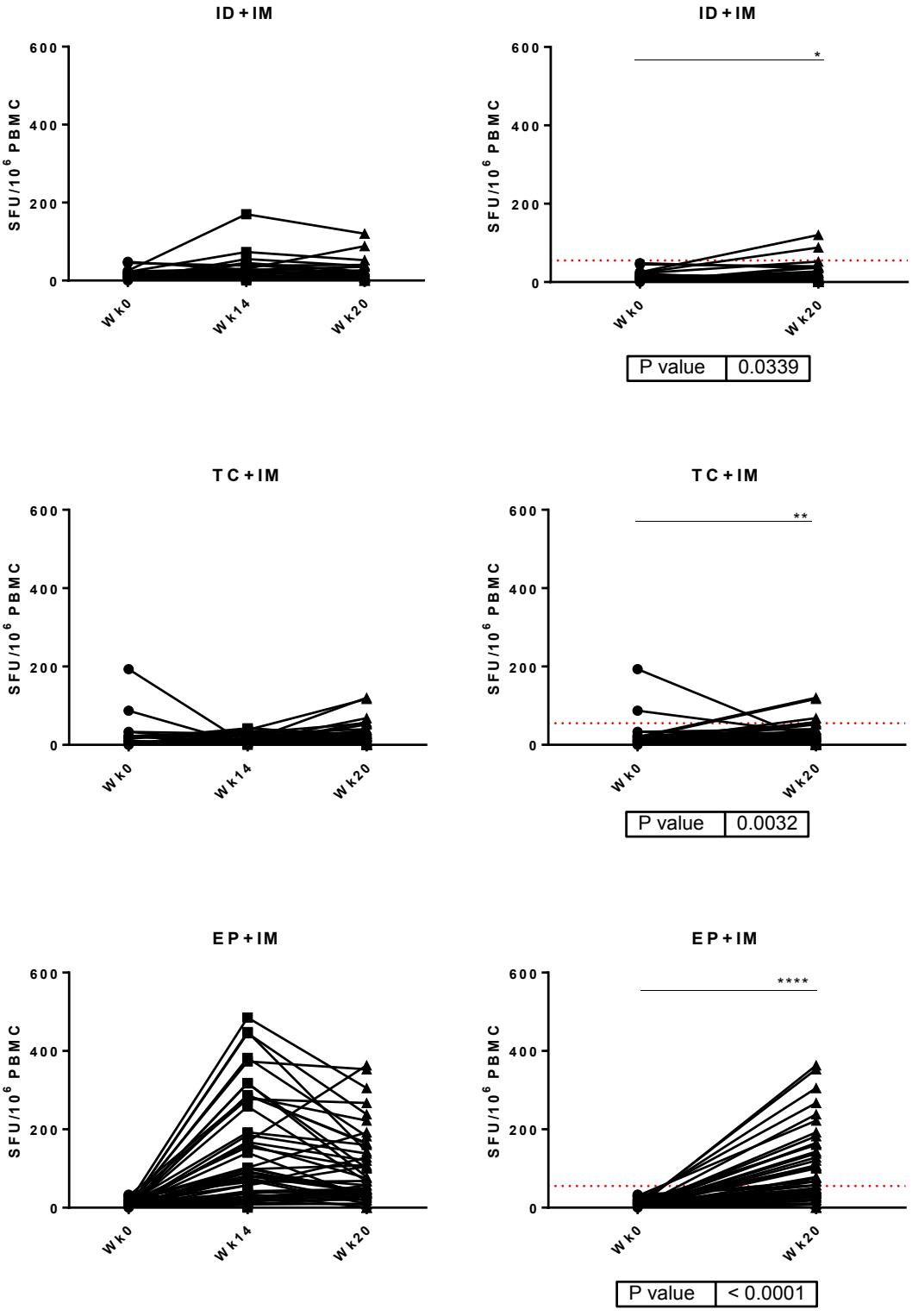


Figure 4.14: Before-after graphs showing all participants' IFN-ɣ response at week 0, 14 and 20, with results from Wilcoxon matched-pairs signed rank test demonstrating statistical significance across all three groups between week 0 and week 20.

Summary of Immunogenicity results: T cell IFN-ɣ ELISpot responses

This section demonstrates that DNA vaccination induced HIV-specific immunity in HIV uninfected volunteers and that the T cell response was influenced by the route of vaccine administration.

- Electroporation preferentially expands IFN-ɣ responses as the primary end point, displaying the greatest number of responders and the greatest magnitude of response in this group
- IFN-ɣ responses across the interim time points (weeks 1 to 12) are variable but there are a consistently greater number of responders at any time point in the EP+IM group
- Electroporation with IM vaccination showed the greatest breadth of the IFN-ɣ response across all time points
- The duration of the IFN-ɣ response was greatest in the EP+IM group out to week 20
- In summary: The EP+IM group showed a greater magnitude, breadth and duration of response across all peptides at the primary end point and interim time points by IFN-ɣ T cell ELISpots compared to the other groups

4.3 SUPPORTING WORK: DATA ANALYSIS FROM ICS

To study the quality of the cellular immune response, further analysis is of the results of vaccine-induced T cells by ICS stimulated with the same peptide pools.

Data analysis from the ICS results is presented comparing week 0 (baseline) to the primary end point. The ICS data is presented as a percentage total of CD4+ or CD8+ cells with all background responses from negative controls subtracted. For the CD4+ results data for cytokines IFN-ɣ, IL-2, TNF-α and CD154 is presented, and for CD8+ cytokines IFN-ɣ, IL-2, TNF-α, CD57, granzyme B and perforin.

4.3.1 No distinct pattern of response is evident at the primary end point comparing all three groups for either CD4+ or CD8+

At week 14, the CD4+ ICS data for IFN-ɣ shows although there appears to be a trend in higher responses in the EP+IM group for all peptides, a significant difference was only present in the *gag*-specific IFN-ɣ response in EP+IM compared to ID+IM (p=0.0052) and EP+IM compared to TC+IM (0.0302) (Figure 4.15). Similarly for IL-2 a significant difference was only found in the *CTL*-specific response between TC+IM and ID+IM groups (p=0.0053) (Figure 4.16). There were no other significant responses between groups to any peptide for TNF-α or CD154 (Figures 4.17 and 4.18 respectively).

For the CD8+ data, the ICS responses appear more evenly distributed across all three groups, but none reached statistical significant between groups for any cytokine (Figures 4.19-4.24).

4.3.2 The CD4+ response predominates within each group at the primary end point

CD4+

Comparisons of pre-vaccination (week 0) and primary end point data (week 14) within each group showed for IFN-ɣ there was a significant *Gag*-specific response in the EP+IM group (p=0.0039) not present in other groups or for other peptides (Appendix 6.3). For IL-2 there was a significant difference in the TC+IM group to *Rev* (p=0.0059) (Appendix 6.4), and for TNF-α in the ID+IM group to *Nef* (p=0.0391) and the EP+IM group to *Rev* (p=0.0137) and *Gag* (p=0.0020) (Appendix 6.5 and 6.6). No differences were found in any group to any peptide for CD154.

CD8+

Comparisons of pre-vaccination (week 0) and primary end point data (week 14) within each group showed for IFN-ɣ there was a significant difference in *Rev* (p=0.0391) and *CTL*-specific responses (p=0.0273) in the EP+IM group but not in other groups (Appendix 6.7). There were no differences in any group to any peptide for perforin, IL-2, TNF-α, CD57 or granzyme B at these time points.

4.3.3 The mean pooled peptide response at week 14 in the TC+IM group for all cytokines is comparable in magnitude and breadth to the EP+IM group

CD4+

The mean pooled peptide response for IFN-ɣ follows a similar pattern in the ICS data as for the ELISpot data. However, this pattern is not reflected in the other cytokines (IL-2, TNF-α, and CD154), emphasizing the importance of the ICS data in assessing the overall qualitative immune response. This is particularly evident in the TC+IM group, where the mean pooled peptide response for IL-2, TNF-α, and CD154 is of similar magnitude and breath to the EP+IM group and in some participants exceeds the magnitude seen in the EP+IM group, a pattern that was not evident in the ELISpot results (Figure 4.25). A similar pattern is seen in the ID+IM group for these cytokines.

CD8+

Similarly the CD8+ mean pooled peptide response highlights the TC+IM response in cytokines other than IFN-ɣ, particularly IL-2, TNF-α, CD57 and granzyme B which in some participants exceeds the magnitude seen in the EP+IM group (Figure 4.26).

4.3.4 Correlation between ELISpot and ICS data

Finally, to determine whether there was a correlation between my T Cell ELISpot IFN-ɣ data and the IFN-ɣ results from the ICS, a correlation analysis of the week 14 data from the IFN-ɣ ELISpot assay and the combined CD4+/CD8+ ICS data for IFN-ɣ was conducted.

In the ID+IM group, the data shows a statistically significant result for the *Gag* peptide pool (p=0.0166), with a moderately positive correlation coefficient (R^2=0.5836). There was no correlation found between these 2 assays for the remaining peptide pools (Figure 4.27). In the TC+IM group there was no relationship found between IFN-ɣ ELISpots and the ICS data for any peptide pool (Figure 4.28).

In the EP+IM group there was a highly statistically significant relationship for the *Nef* peptide pool ($p=0.0004$) with a strong positive correlation coefficient ($R^2=0.8097$). Although, the best fit line for the *Gag* and *CTL* peptide pools also indicated a positive relationship these did not reach statistical significance (Figure 4.29).

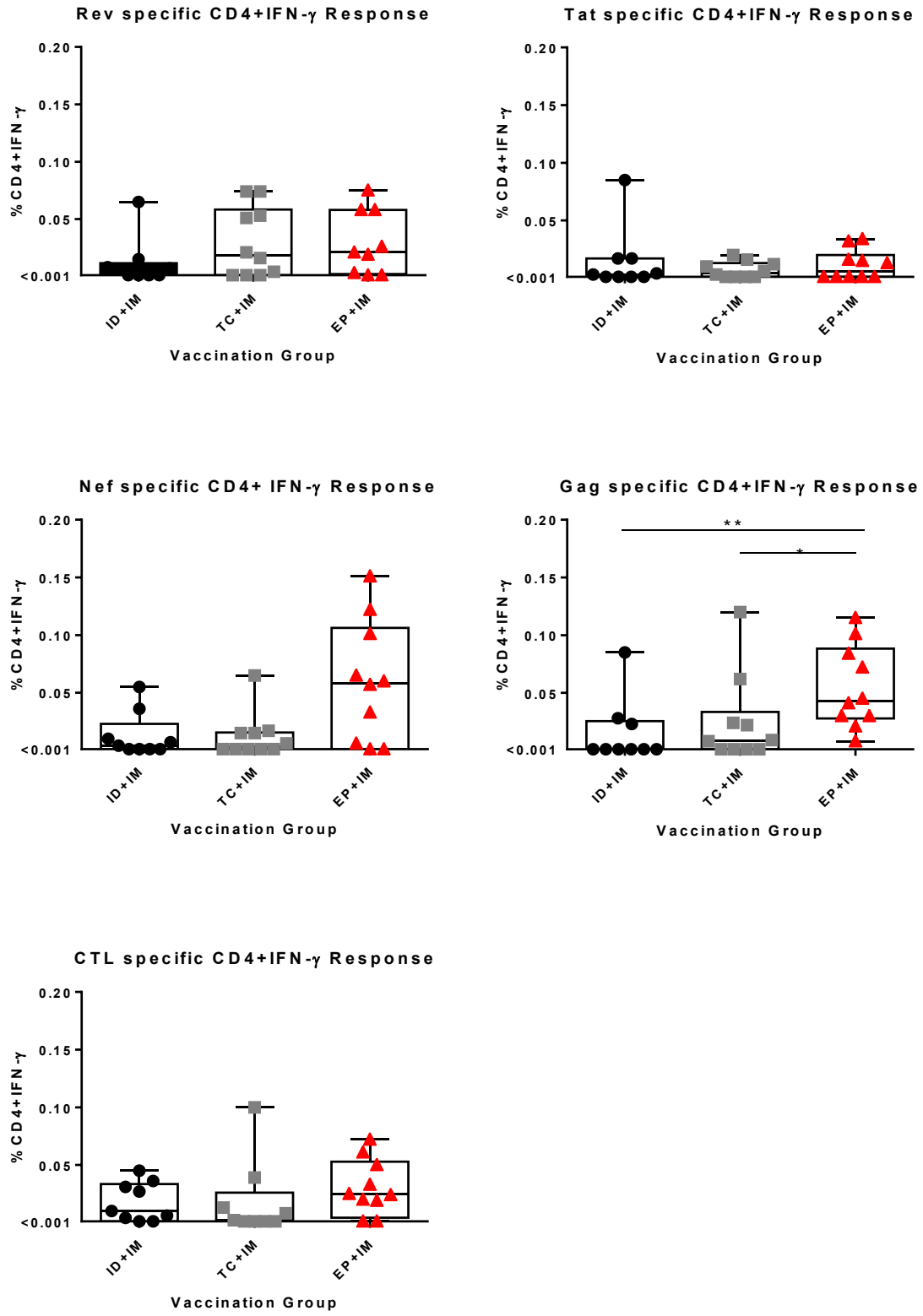


Figure 4.15. CD4+ IFN-ɣ ICS data showing HIV specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05

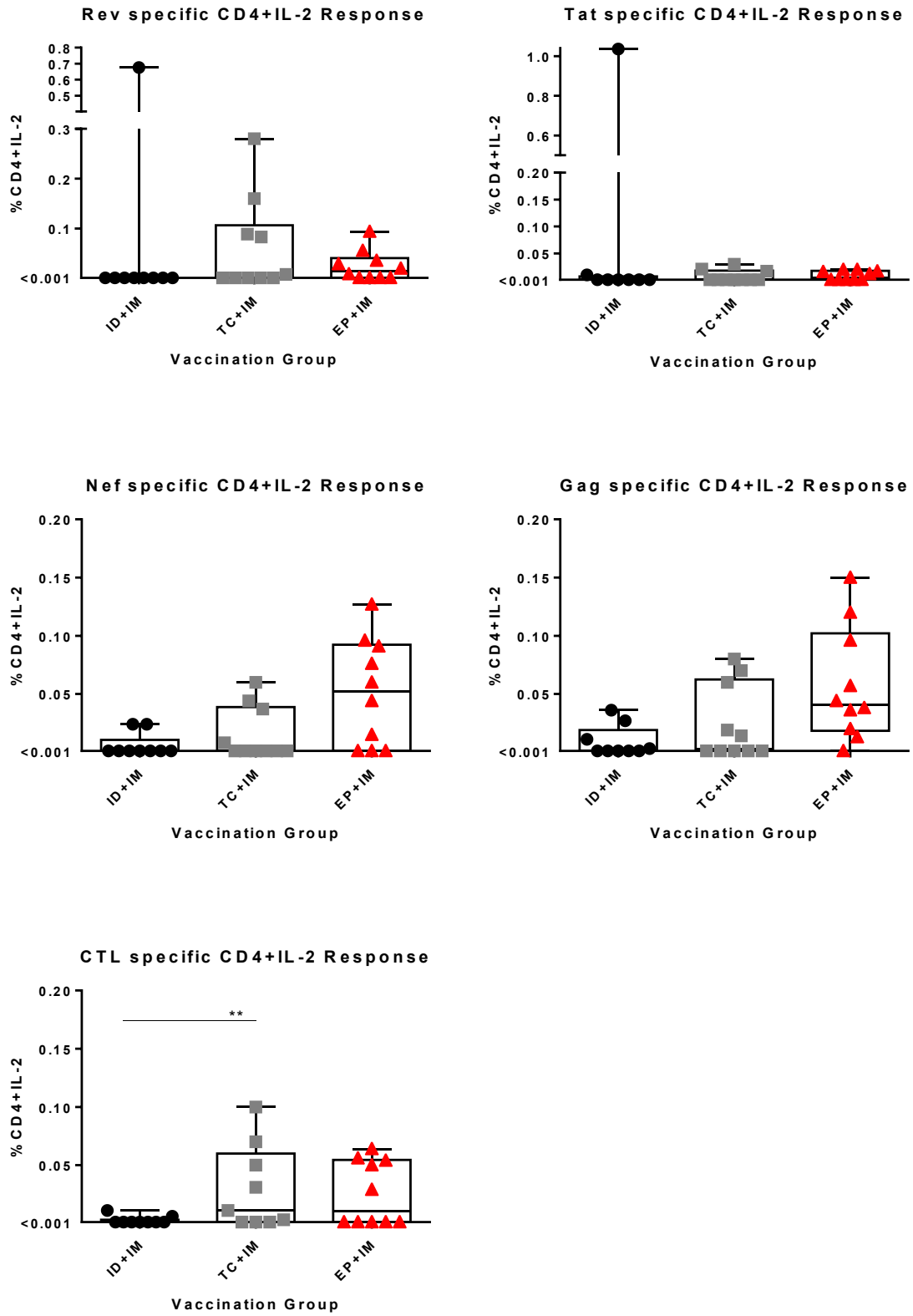


Figure 4.16. CD4+ IL-2 ICS data showing HIV specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05

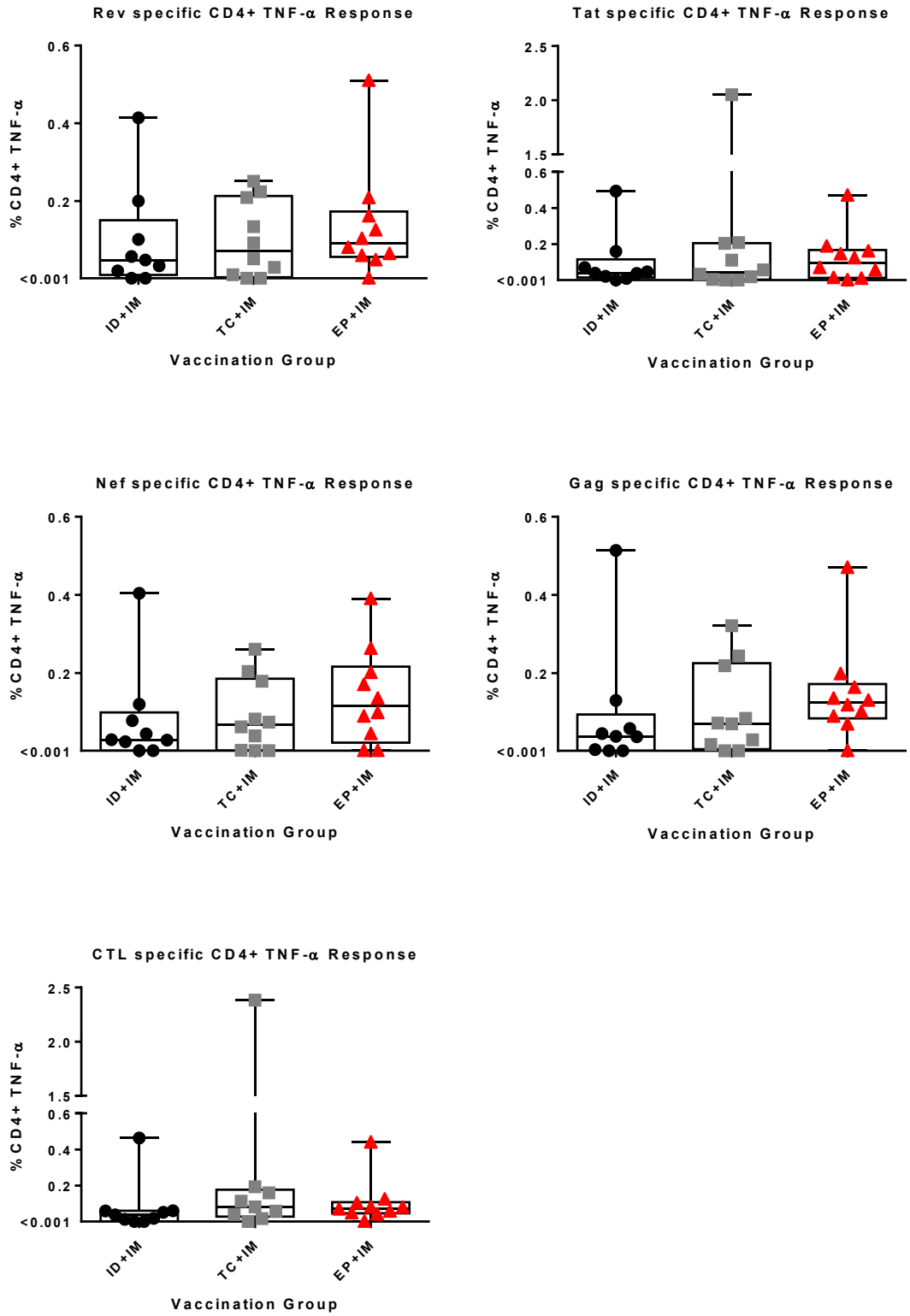


Figure 4.17. CD4+ TNF-α ICS data showing HIV specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05

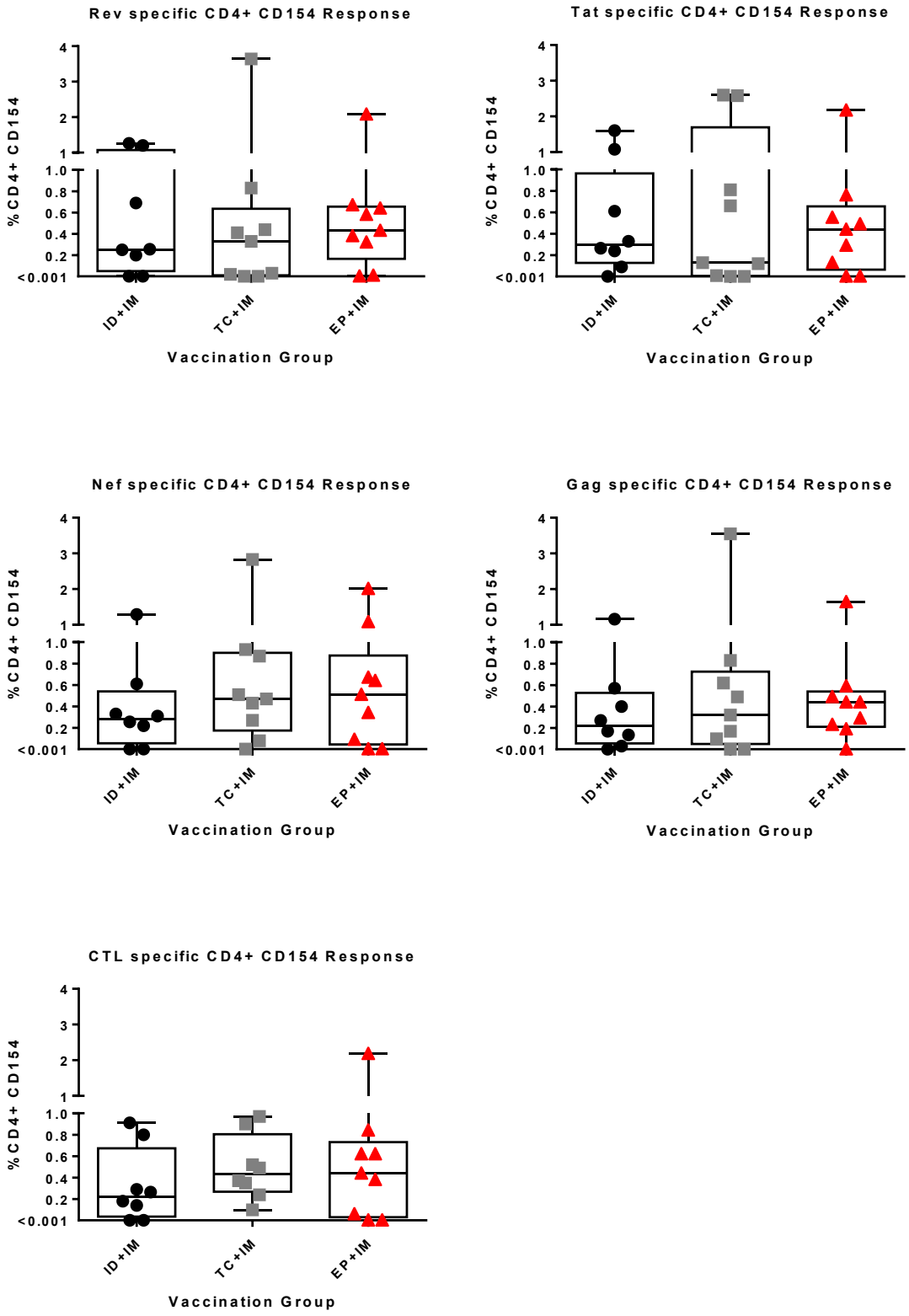


Figure 4.18. CD4+ CD154 ICS data showing HIV specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05

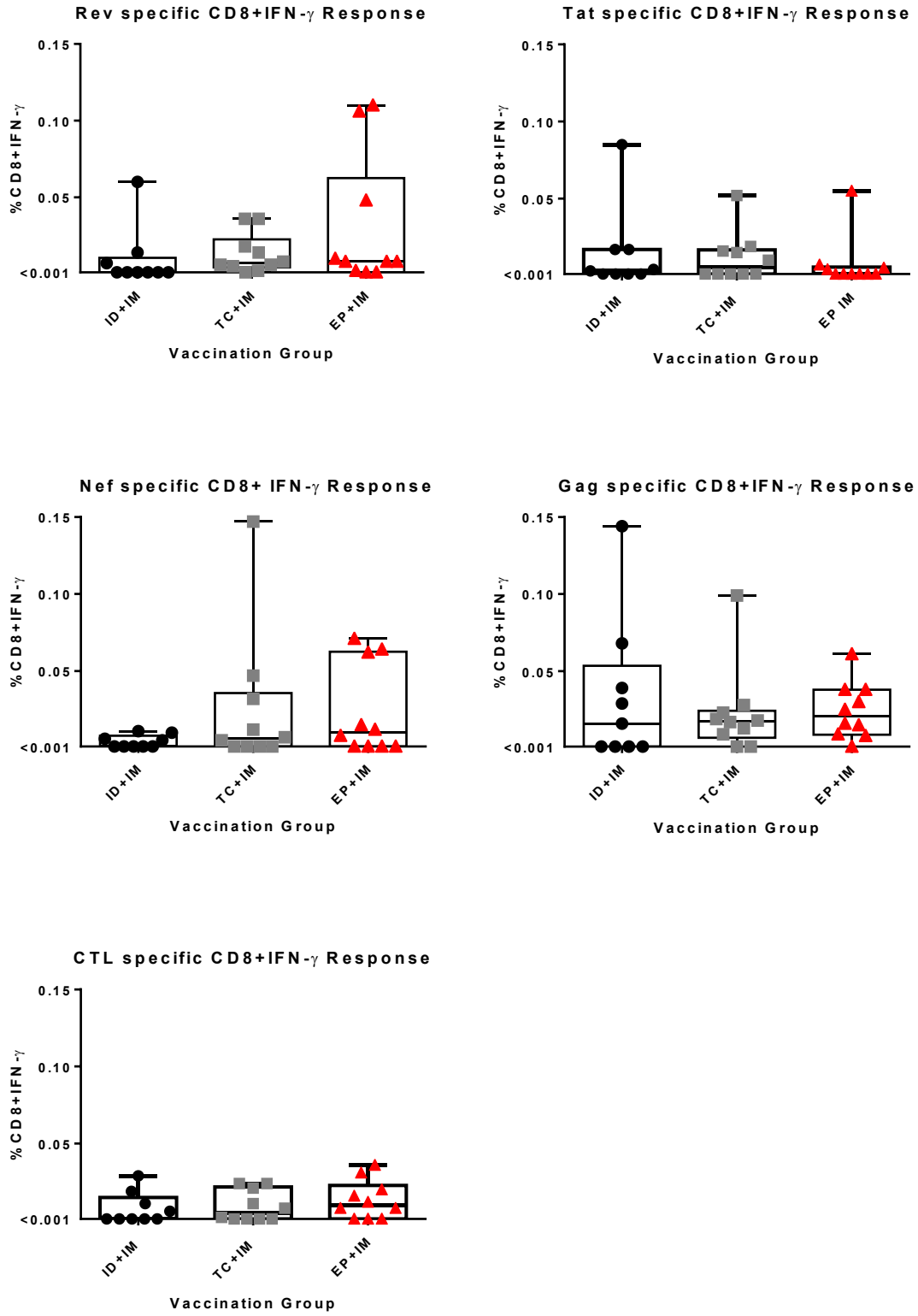


Figure 4.19. CD8+ IFN-ɣ ICS data showing HIV-specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05.

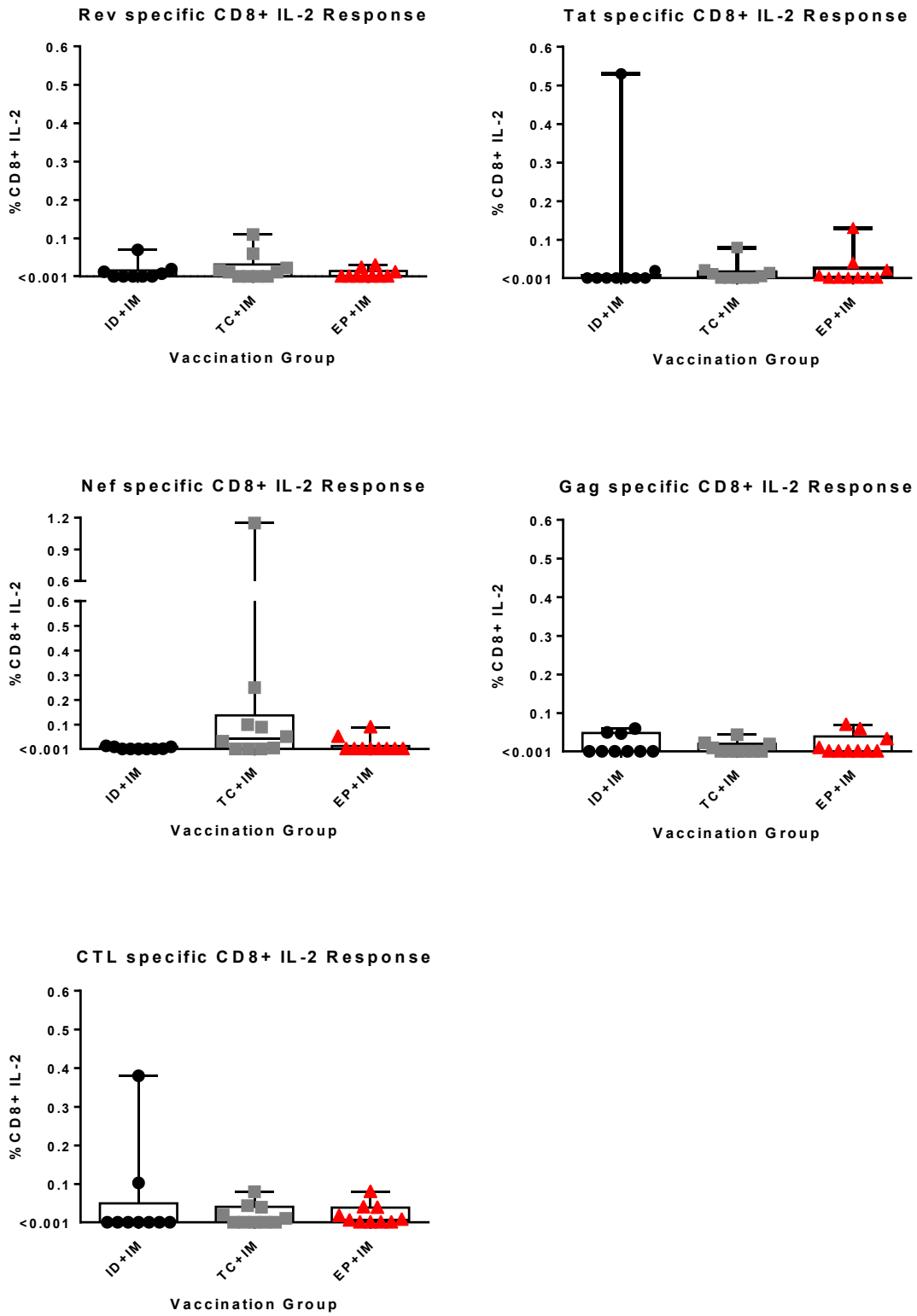


Figure 4.20. CD8+ IL-2 ICS data showing HIV-specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05.

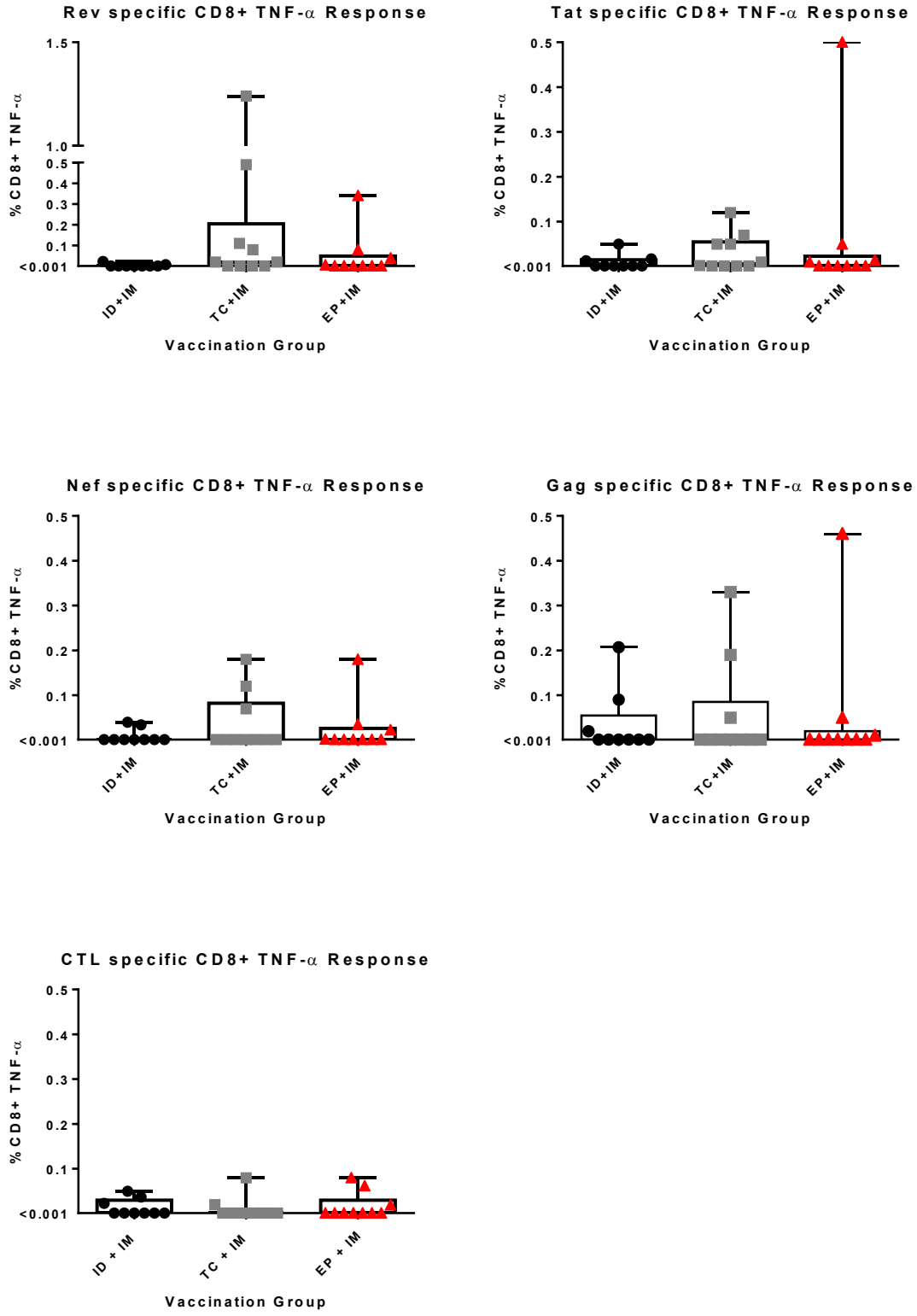


Figure 4.21. CD8+ TNF-α ICS data showing HIV-specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05.

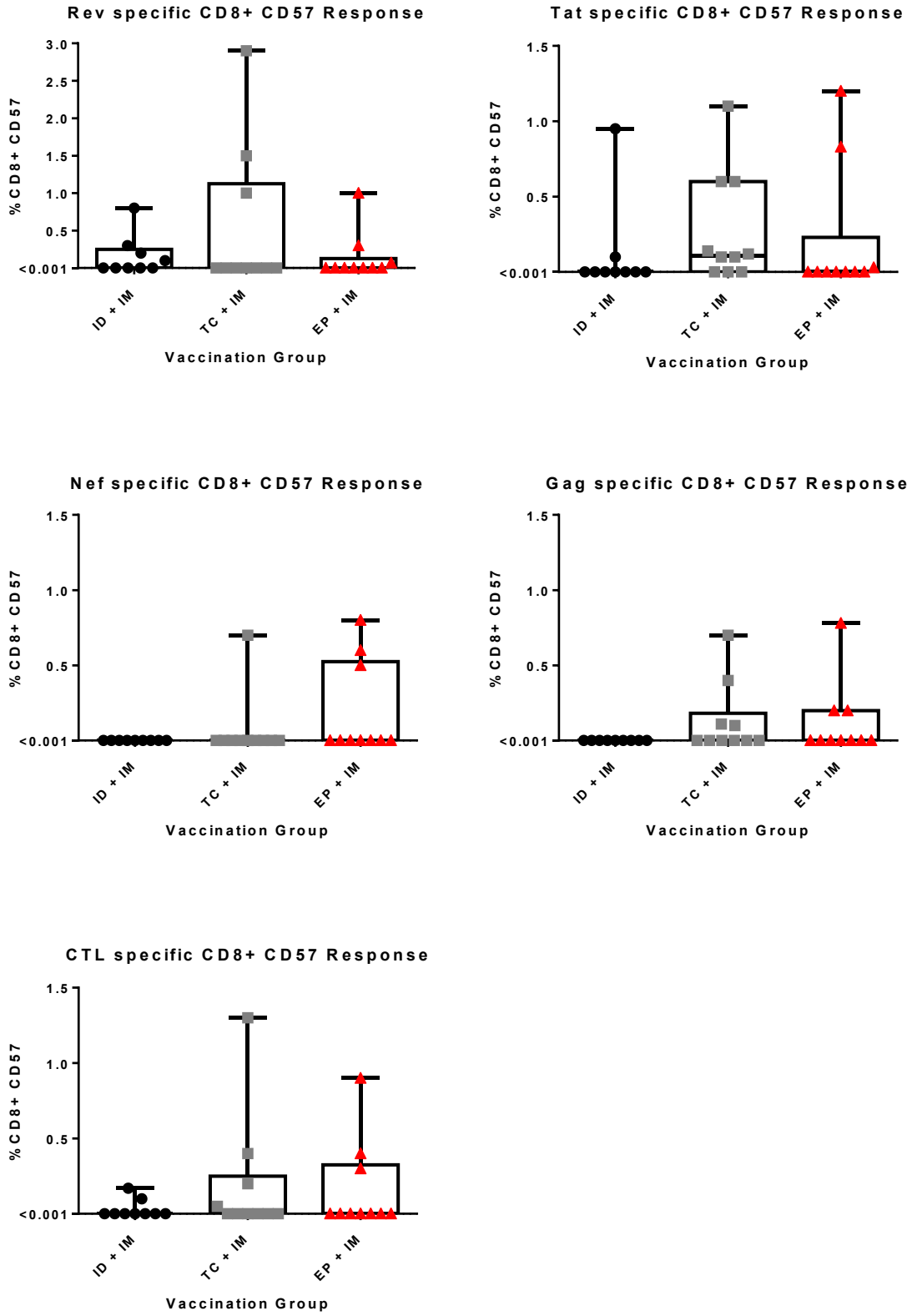


Figure 4.22. CD8+ CD57 ICS data showing HIV-specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05.

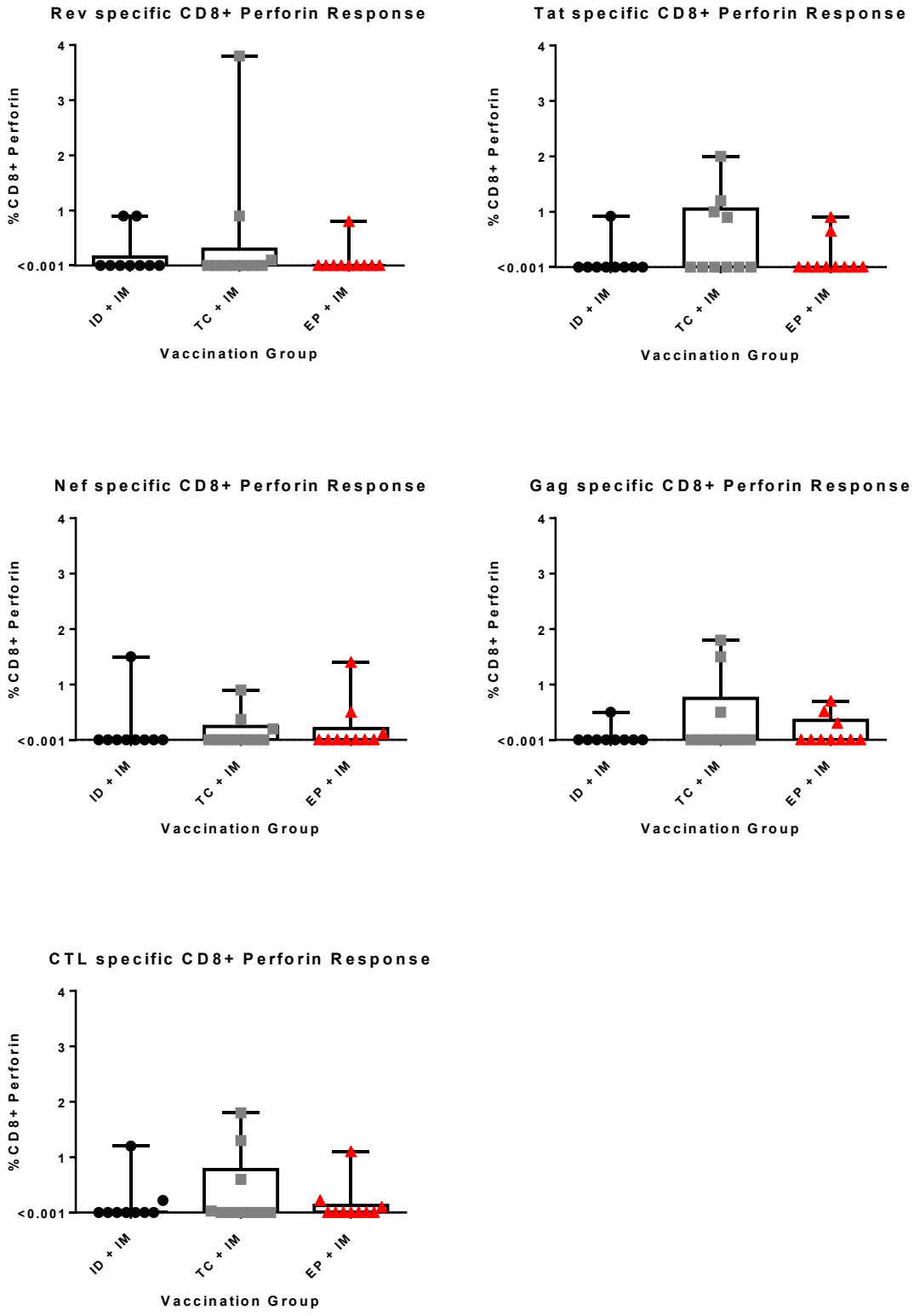


Figure 4.23. CD8+ Perforin ICS data showing HIV-specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05.

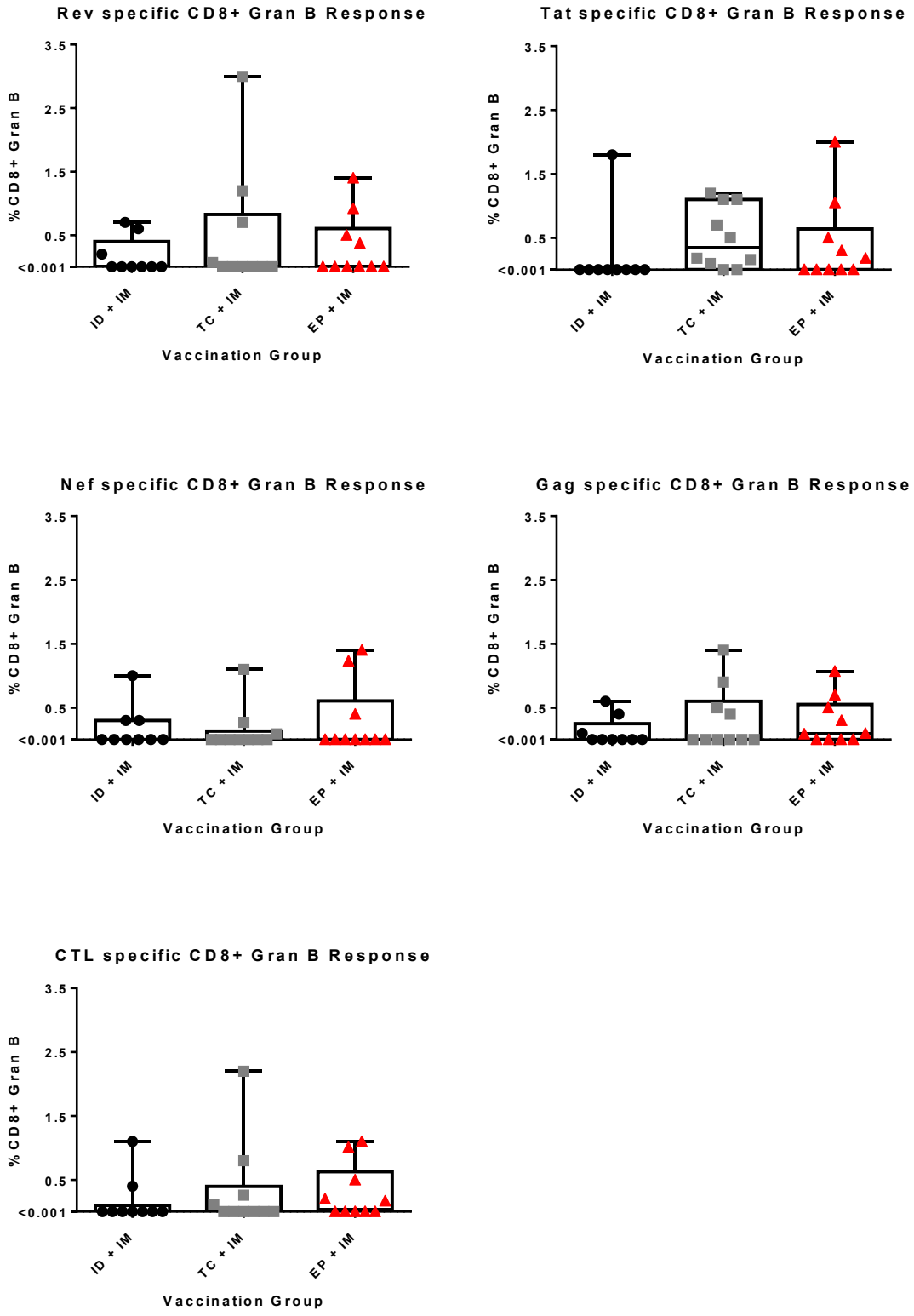


Figure 4.24. CD8+ Granzyme B ICS data showing HIV-specific responses at week 14 comparing all 3 groups. Statistical significance was set at p=<0.05.

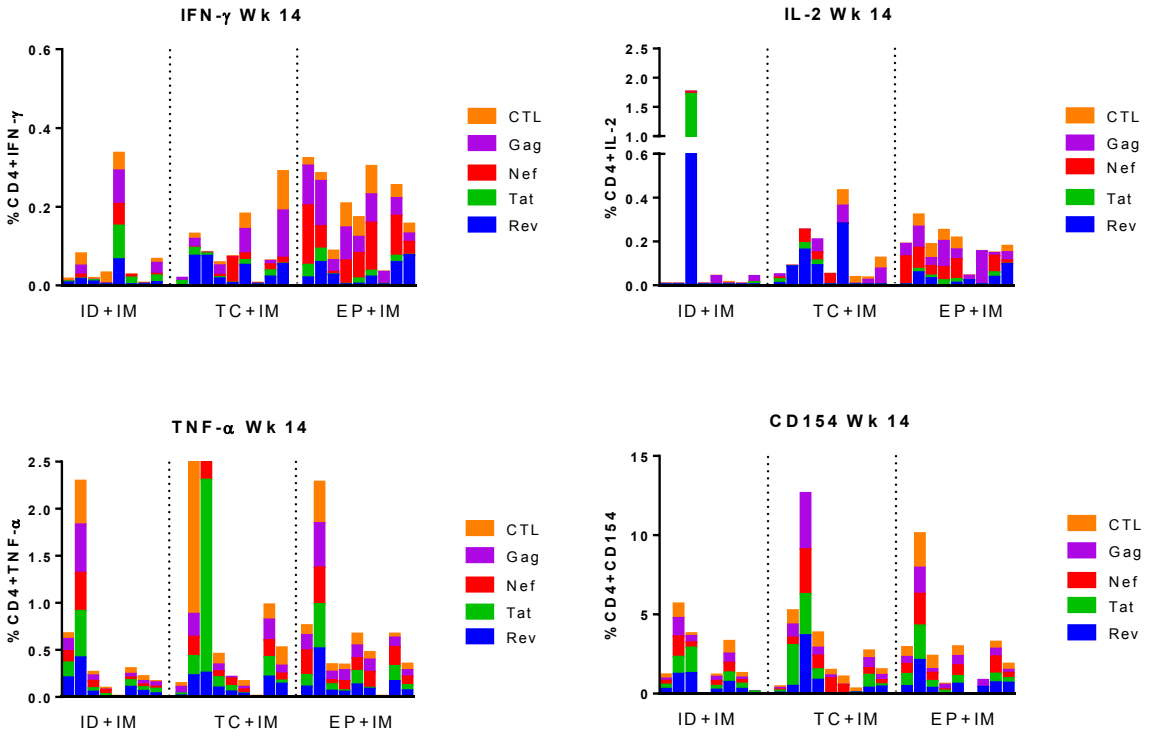


Figure 4.25: CD4+ mean pooled peptide response at week 14. The results are presented as percentage (%) of CD4+ cells expressing the cytokine. Each stacked bar represents the mean response per peptide pool for one participant. The breadth of the response for each participant is shown by colour (matched to peptide pool).

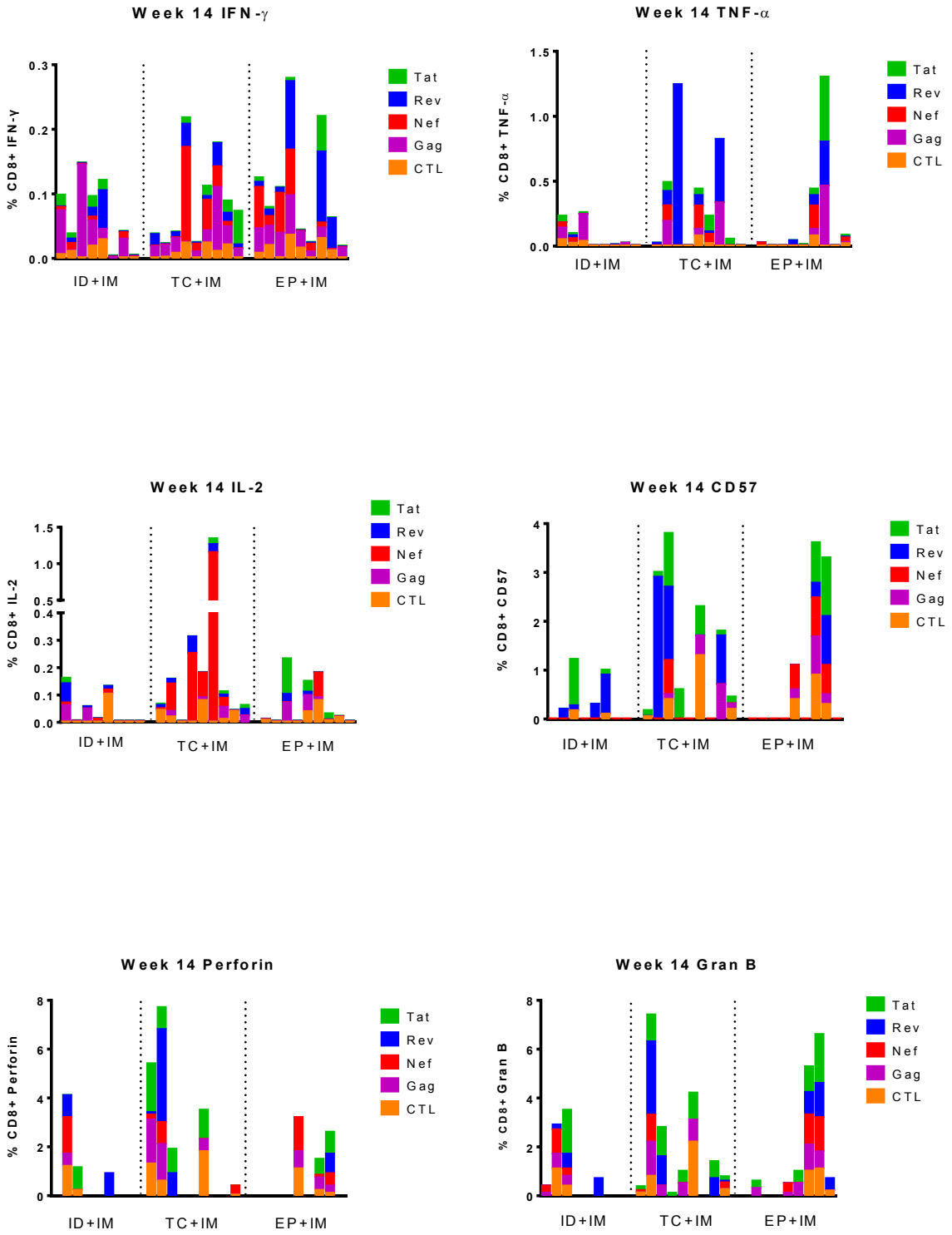


Figure 4.26: CD8+ mean pooled peptide response at week 14. The results are presented as percentage (%) of CD8+ cells expressing the cytokine. Each stacked bar represents the mean response per peptide pool for one participant. The breadth of the response for each participant is shown by colour (matched to peptide pool).

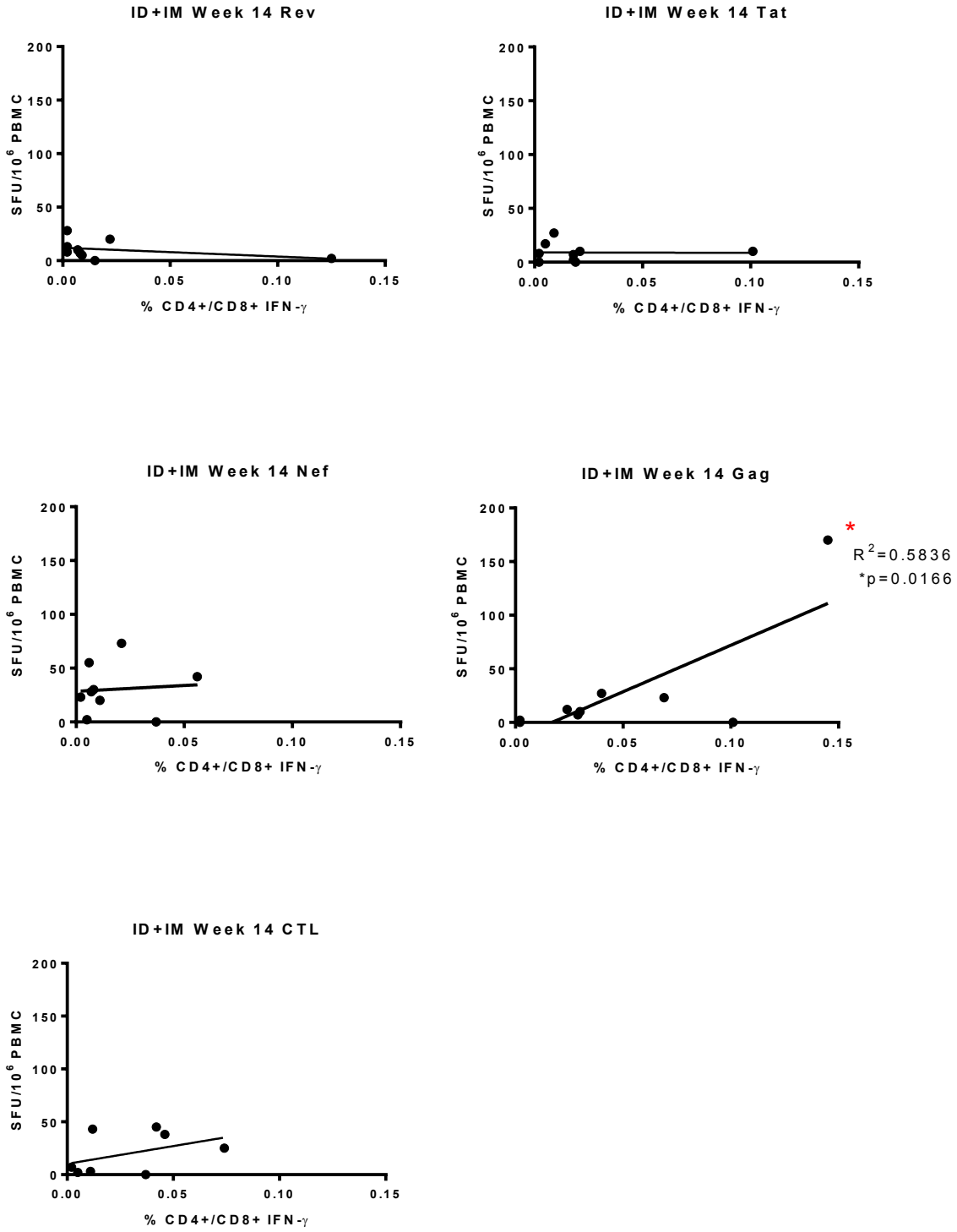


Figure 4.27: Correlation analysis for the ID+IM group at week 14 examining the relationship between the T cell ELISpot IFN-ɣ results on the y axis and the pooled CD4+/CD8+ ICS data for IFN-ɣ for the x axis for each peptide pool. Where appropriate, the p value and correlation coefficient (R^2) is shown indicating a positive relationship between the 2 assays.

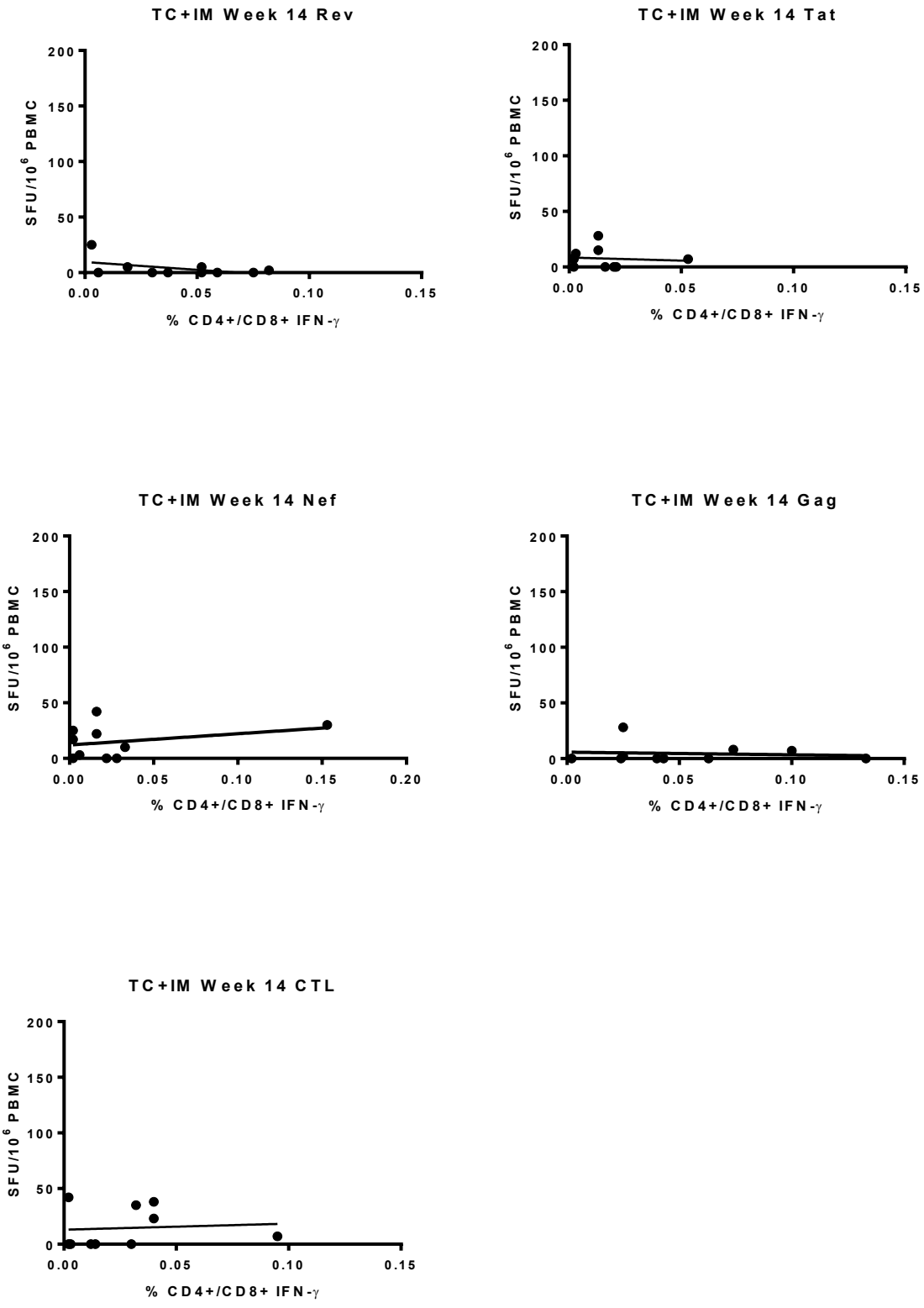


Figure 4.28: Correlation analysis for the TC+IM groups at week 14 examining the relationship between the T cell ELISpot IFN-ɣ results on the y axis and the pooled CD4+/CD8+ ICS data for IFN-ɣ on the x axis for each peptide pool. Where appropriate, the p value and correlation coefficient (R^2) is shown indicating a positive relationship between the 2 assays.

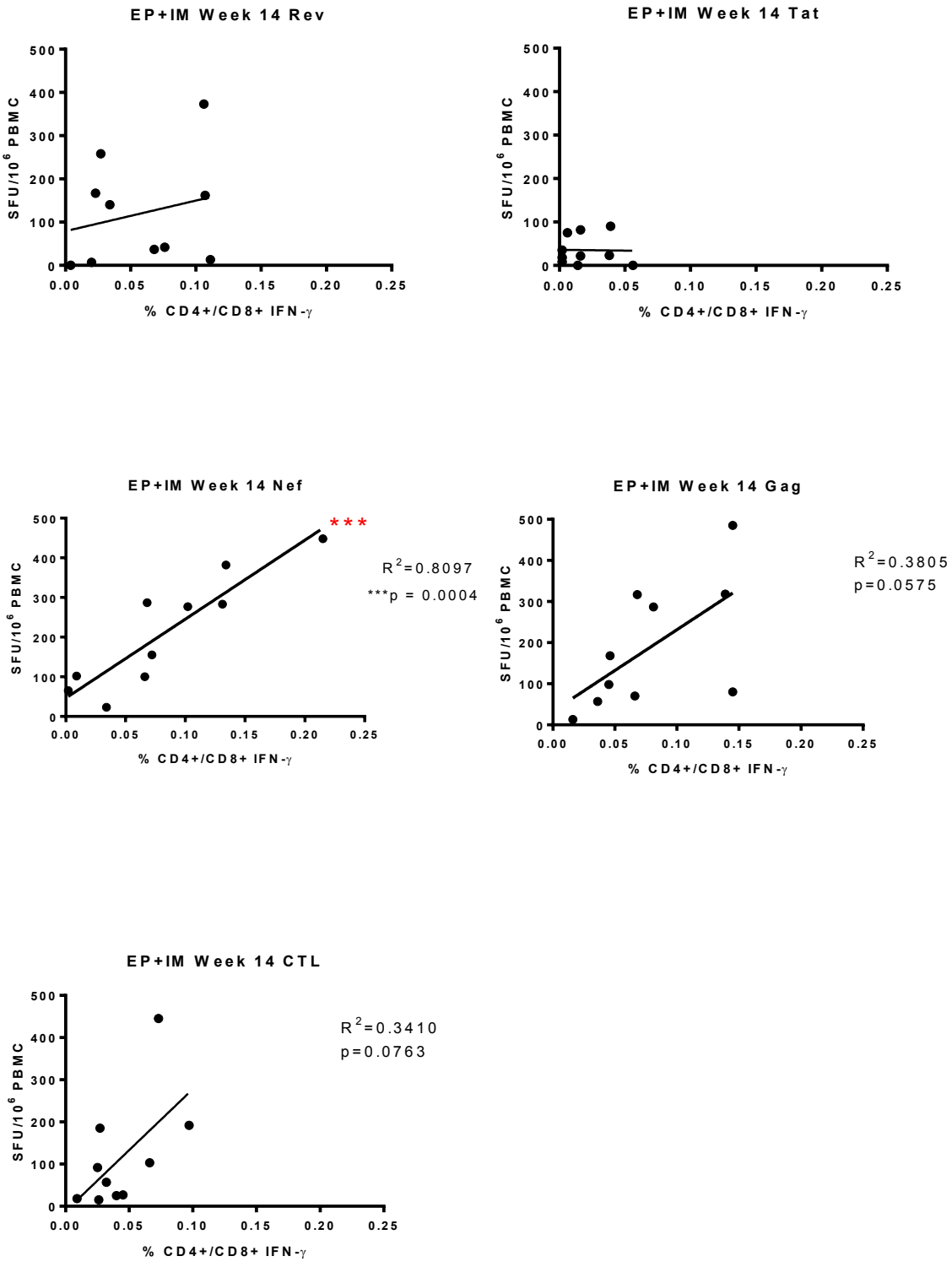


Figure 4.29: Correlation analysis for the EP+IM groups at week 14 examining the relationship between the T cell ELISpot IFN-ɣ results on the y axis and the pooled CD4+/CD8+ ICS data for IFN-ɣ on the x axis for each peptide pool. Where appropriate, the p value and correlation coefficient (R^2) is shown indicating a positive relationship between the 2 assays.

Summary of Immunogenicity results: ICS

- The ICS data highlights a predominantly CD4+ driven response at the primary end point in all three groups
- The mean pooled peptide response at week 14 in the TC+IM group for all cytokines is comparable in magnitude and breadth to the EP+IM group
- The EP+IM group showed the greatest correlation between the ELISpot and ICS results
- In summary: The ICS data shows a different pattern in response to several cytokines, which could not have been predicted by IFN-ɣ alone, in particular suggesting transcutaneous vaccination may modulate the quality and function of induced T cell responses

SECTION B: VIRAL INHIBITION ASSAY CUTHIVAC 001

4.4 INTRODUCTION

Although intracellular cytokine staining (ICS) and IFN-ɣ ELISpot assays are routinely employed to assess vaccine immunogenicity, there is ongoing debate over whether ELISpot responses have a direct correlation with *in vivo* virus control. Many studies have questioned whether greater importance should be placed on T cell functionality [222]. One such study examining HIV specific T cell responses during acute infection aimed to establish whether T cell responses in primary infection could correlate with viral set point and be used as a marker of disease progression [223].

In this study 47 clade C HIV-1 infected participants from the CAPRISA 002 cohort were recruited within a mean of 6.1 weeks of infection. IFN-ɣ responses across the complete viral proteome were measured at a median of 12 weeks post infection. Results showed neither the magnitude nor the breadth or responses to any region in the genome using the IFN-ɣ assay during early infection predicted the course of viraemia, early disease progression or the set point. This raises the question of whether the use of IFN-ɣ is adequate for assessing the impact of T cell immunogenicity. Hence these findings suggest that IFN-ɣ may not reflect the status of T cells or their function in controlling viraemia.

The STEP study as discussed in an earlier section, was a phase IIB proof-of-concept trial to determine if the MRKAd5 HIV-1 *gag/pol/nef* vaccine could lower HIV-1 infection rates or post-infection reduce plasma viraemia [101]. Overall, this vaccine elicited a higher CD8+ T cell response rate and magnitude than that reported for any vaccine tested over the past 15 years, yet failed to protect against HIV infection or disease progression in those infected. This study raises fundamental questions on how to move the HIV vaccine field forward and how best to measure vaccine efficacy, indicating the presence of T cell INF-ɣ responses alone independent of magnitude or breadth of response may not correlate to protection.

The function of CD8+ cells can be examined more closely using the viral inhibition assay (VIA), with an arguably better prediction of *in-vivo* virus control. Since 1997, the VIA has been modified and developed. The assay is no longer limited to specific HLA types, and bi-specific antibodies are now used for cell expansion, making the assay practical when cell numbers are limited. CD3/4 and CD3/8 bispecific monoclonal antibodies cause a profound decrease in their respective CD8+ and CD4+ populations. Although the mechanism of action is not entirely understood, it is proposed a 'bridging' mechanism exists where for example, CD4+ target cells will attach to CD3 complexes of effector cells

using these antibodies (CD3/8 in this example), mirroring cytolytic T cell and target cell interactions [224].

As described in detail in the methods section autologous CD8+ and CD4+ cells are each expanded separately with the respective bispecific antibodies in order to evaluate anti-viral functions of CD8+ lymphocytes. Exogenous virus is added to infect CD4+ cells and an objective method of p24 measurement using ELISA is used to evaluate inhibitory activity [225].

The VIA I conducted in this trial compares inhibition in PBMC samples from baseline (week 0) and the primary end point (week 14) assessed against a panel of 6 viruses: 3 clade B (IIIB, CH077, CH106) and 3 cross clade viruses (U455 clade A, CBL4 clade D, and ELI clade A/D) (Table 2.4 in Methods Chapter 2). In this 3 week assay, contamination of cultures can occur, and for this reason the following participants had to be excluded for analysis as the assay could not be completed: TC+IM- 2 participants (C009, C052); EP+IM- 3 participants (C011, C029, C053). The cut-off for a positive response was defined as >1.5 $\log^{10}$ and >0.6 $\log^{10}$ difference between week 14 and week 0.

In this section the results from the VIA are presented which is relevant to my second hypothesis and aim in HIV negative volunteers.

4.5 RESULTS: VIRAL INHIBITION ASSAY

4.5.1 All groups exhibited viral inhibition at the primary end point with the greatest inhibition in the EP+IM group, with evidence of cross-clade inhibition

Although a clade B vaccine construct was used in this trial, interestingly there were a greater number of responders at week 14 to the non-clade B viruses, in particular to U455 and CBL4 (Figure 4.30A). However, broken down by group this was not found to be statistically significant (p=0.1218) (Figure 4.30B).

In all groups there were participants exhibiting viral inhibitory activity to at least 1 virus at the primary end point in 2/9, 4/9, and 5/7 participants in ID+IM, TC+IM and EP+IM respectively (Table 4.4). In the ID+IM both responders inhibited 1 virus each. In TC+IM there were a total of 4 responders, 3 of which only responded to 1 virus each and the final participant responding to 3 viruses in total. In EP+IM a total of 5 responders inhibited a minimum of 1 virus, with one participant inhibiting 4 viruses at week 14 (Table 4.5).

Table 4.4: Table to show the number of participants meeting criteria for viral inhibition, with median and maximum log^{10} inhibition shown.

	ID+IM	TC+IM	EP+IM
No. Volunteers	9	9	7
Median Log^{10} Inhibition	1.09	1.01	1.42
Max Log^{10} Inhibition	1.90	2.31	2.88
VIA Responders	2/9	4/9	5/7
% Response rate	22.2	44.4	71.4

Table 4.5. Table to show the number of viruses and type of virus inhibited at week 14 by group and participant.

POSITIVE RESPONDERS ID+IM	**No. Viruses Inhibited**	**Virus inhibited**
C024	1	CH077
C051	1	CBL4
POSITIVE RESPONDERS TC+IM		
C042	1	CH077
C045	1	CBL4
C048	1	CBL4
C059	3	IIIB; CH077; CBL4
POSITIVE RESPONDERS EP+IM		
C021	3	IIIB; U455; CBL4
C030	3	IIIB; U455; CBL4
C036	4	IIIB; ELI; U455; CBL4
C037	1	U455
C049	2	U455; CBL4

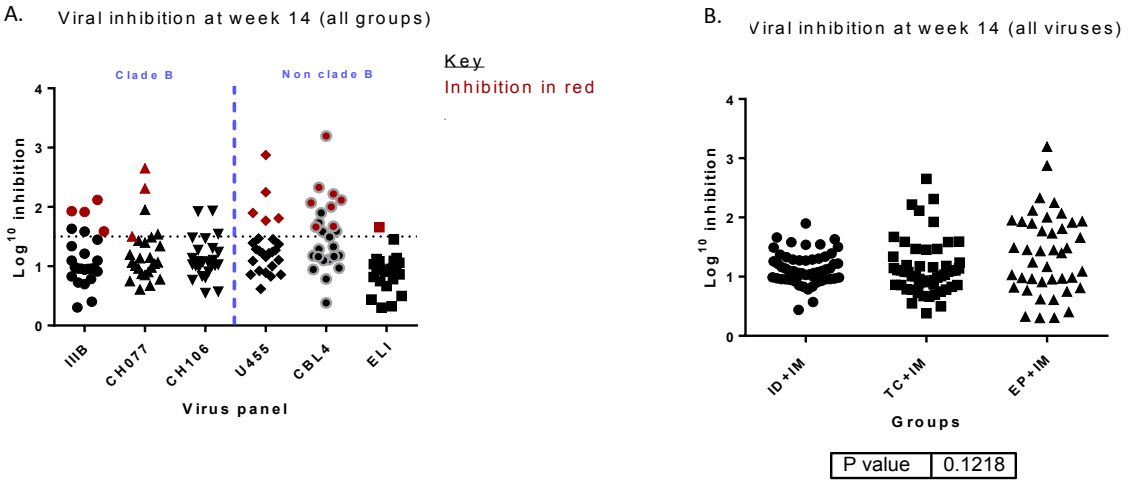


Figure 4.30 A: Graph showing log[10] inhibition of virus panel at week 14, all groups included. Participants showing inhibition are in red and the dotted line represents the cut off of 1.5 log[10]. B: Graph showing log[10] inhibition at week 14 by group. All viruses included. Statistical significance was set at p=<0.05.

CBL4 (clade D) was the most common virus inhibited with 8 participants displaying inhibition to this virus (1 ID+IM, 3 TC+IM, 4 EP+IM) (Figure 31A). Participants from the EP+IM group showed a greater median number of viruses and breadth of viruses inhibited (Figure 4.31B), with a greater number of participants in this group inhibiting more than one virus (Figure 4.32). However of note, all groups exhibited viral inhibition despite limited immunogenicity by IFN-ɣ at the same time point particularly in the TC+IM group where there were no responses by IFN-ɣ at week 14.

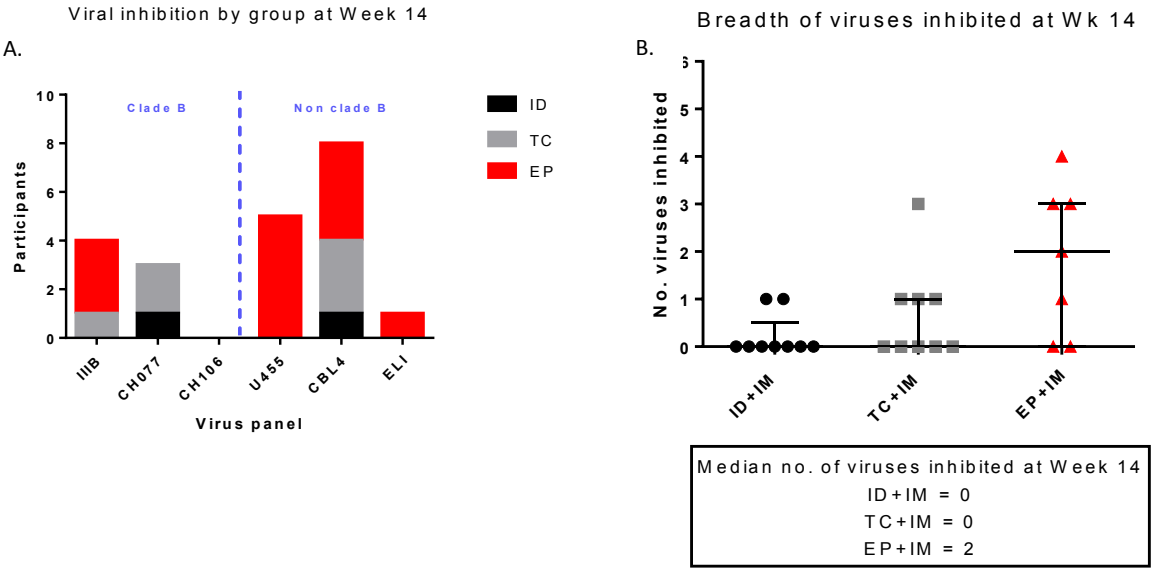


Figure 4.31 A: Viral inhibition by group at week 14 showing the number of participants in each group displaying inhibition (positive responders only) and to which virus in the panel. B: Number of viruses inhibited at week 14 in each group with median number shown.

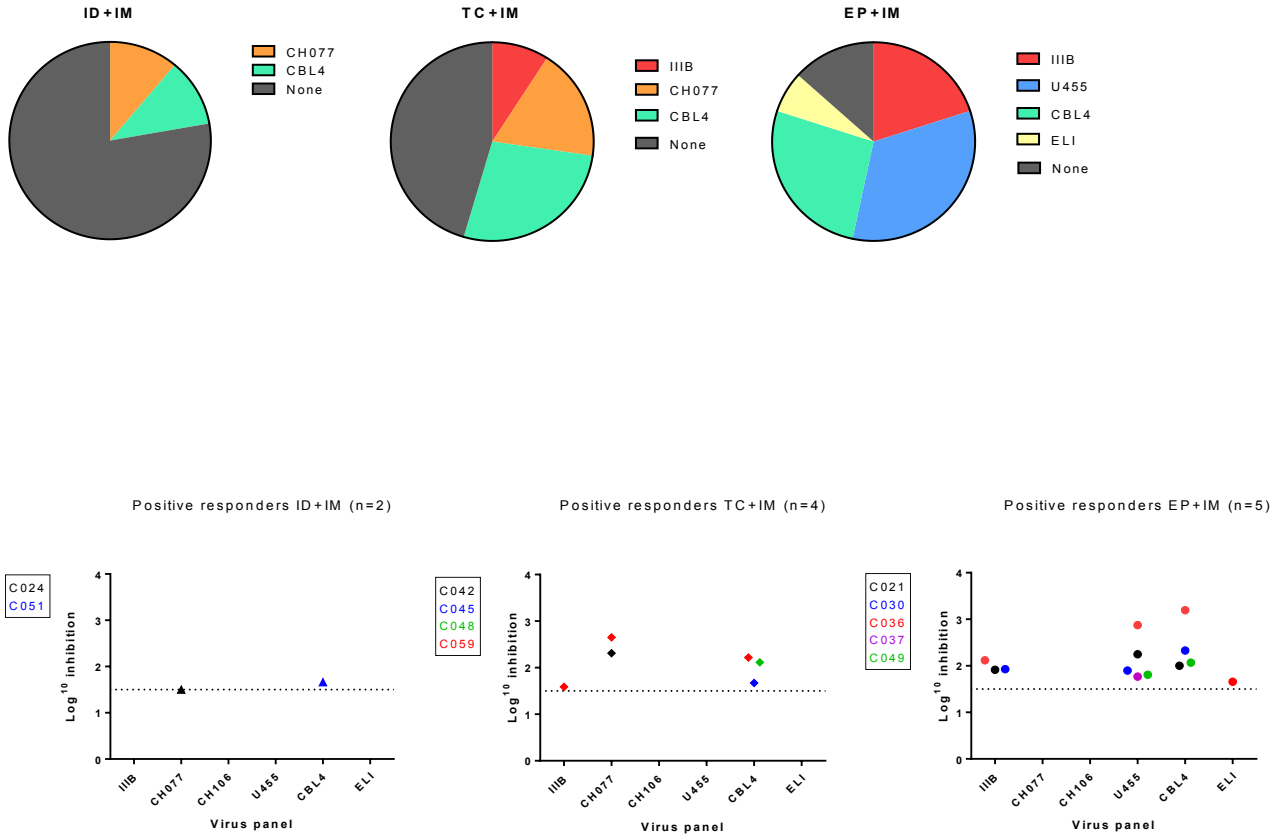


Figure 4.32: Breadth of viral inhibition by group. Pie charts in the upper panel show the number of responders to each virus by group. The graphs in the lower panel show the breakdown of individual responders and how many viruses each participant inhibited.

4.5.2 Primary end point analysis between groups

ID+IM

Although there were only 2 participants showing viral inhibition at week 14, a Wilcoxon matched-pairs signed rank test examining the difference in log^{10} inhibition in this group from week 0 to week 14 (all viruses) showed a statistically significant response (p=<0.0001). Examining each virus individually also showed a difference at week 14 for all viruses with the exception of IIIB (Figure 4.33). This indicates a general increase in log^{10} inhibition from week 0 to week 14 although it remains important to state there were only 2 participants inhibiting virus according to the criteria, therefore the overall relevance of this makes it unlikely to be biologically significant.

TC+IM

In this group similar to ID+IM there was also a difference in log^{10} inhibition in all viruses at week 14 (p=0.0001), but examining each individually this was only found to be true for CH077, CH106 and U455 (Figure 4.34).

EP+IM

In this group, there was again a statistically significant difference found between week 0 and week 14 taking into account all viruses (p=<0.0001), which was seen in all individual viruses excluding CBL4 (Figure 4.35). Examining the positive responders more closely in both assays, in general it can also be said the participants who responded to the most peptide pools at week 14 in the T cell ELISpots, also displayed viral inhibition against the most viruses (Table 4.6), although is not seen in participants C037 and C043.

Table 4.6: EP+IM group table to show the number of peptide pools responded to at week 14 in the T cell ELISpot assay and whether the same participants displayed viral inhibition. NB only 7 participants included (from a total of 10 in this group) as these were the only participants in which the VIA was completed.

Participant EP+IM group	No. peptides pools responded to week 14 (T cell ELISpot)	No. viruses inhibited at week 14 (VIA)
C021	5	3
C030	4	3
C036	5	4
C037	3	1
C043	4	0
C049	3	2
C058	1	0

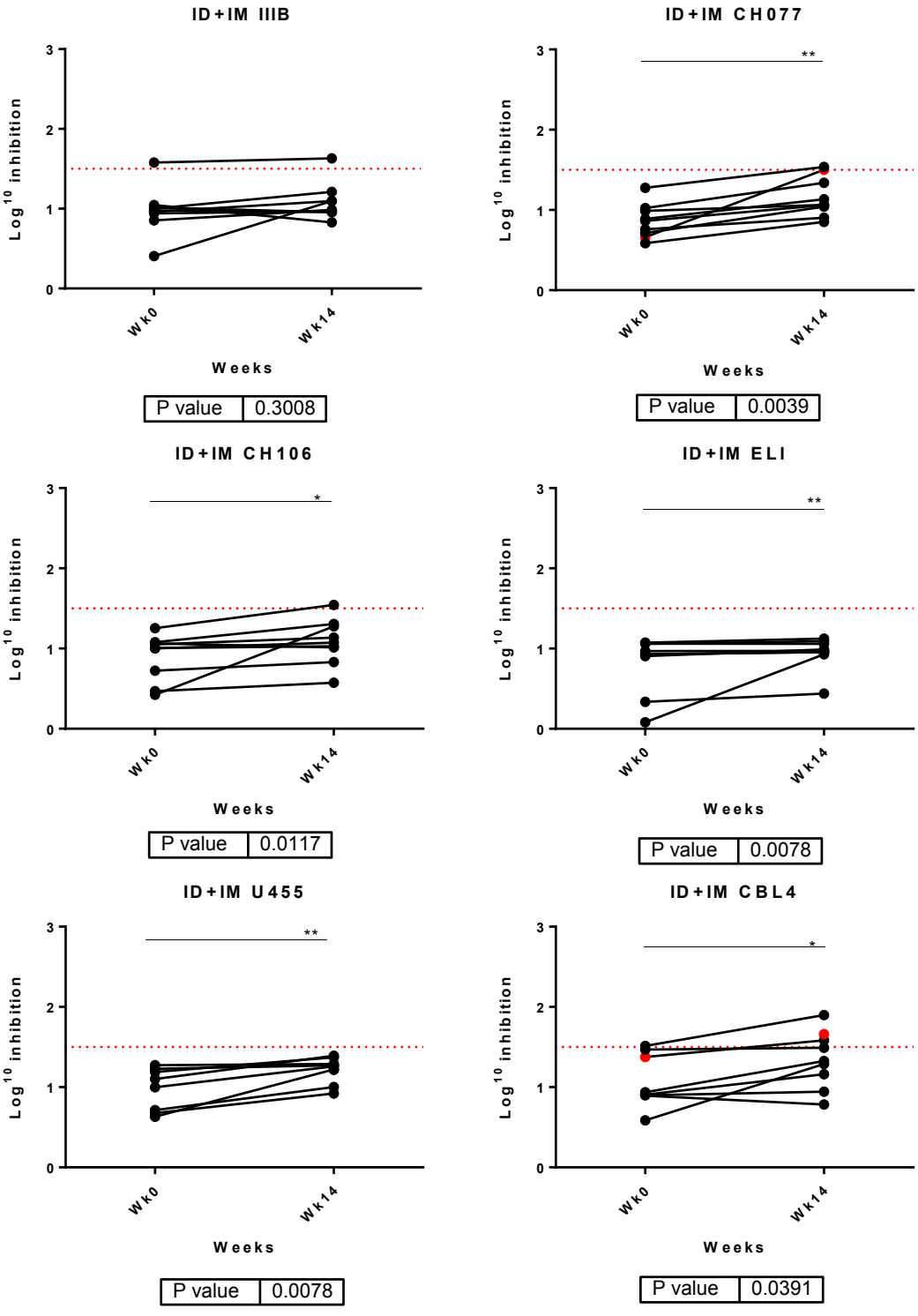


Figure 4.33: Before-after plot for ID+IM group for each individual virus showing week 0 and week 14 log^{10} inhibition, where each dot joined by a straight line is one participant's week 0 and week 14 values. Although statistical significance is shown for some viruses, it is important to note few participants week 14 values are above the 1.5 log^{10} cut-off as shown by the red dotted line. The positive responders are shown in red. Statistical significance was set at p=<0.05.

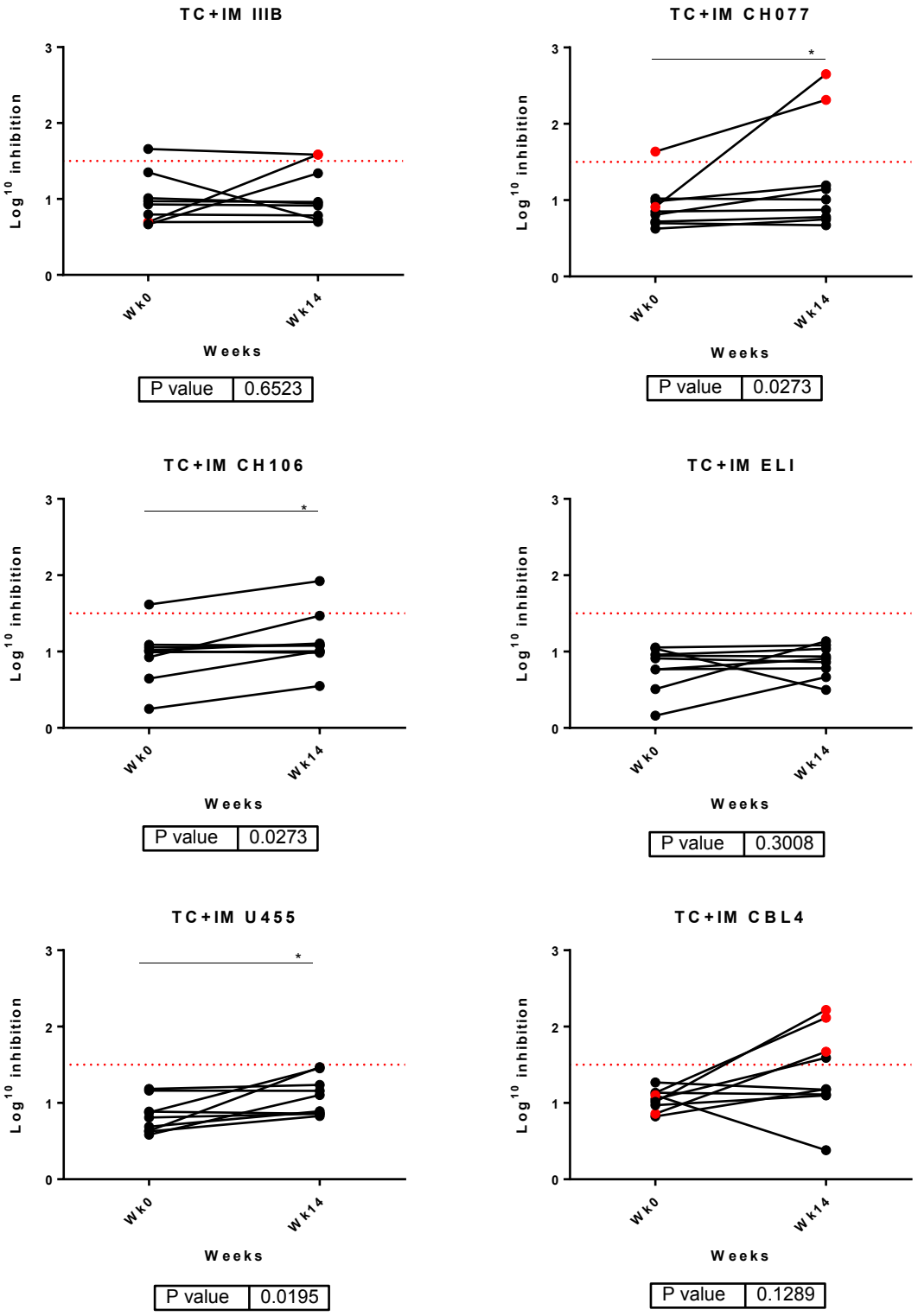


Figure 4.34: Before-after plot for TC+IM group for each individual virus showing week 0 and week 14 log^{10} inhibition, where each dot joined by a straight line is one participant's week 0 and week 14 values. The 1.5 log^{10} cut-off as shown by the red dotted line. The positive responders are shown in red. Statistical significance was set at p=<0.05.

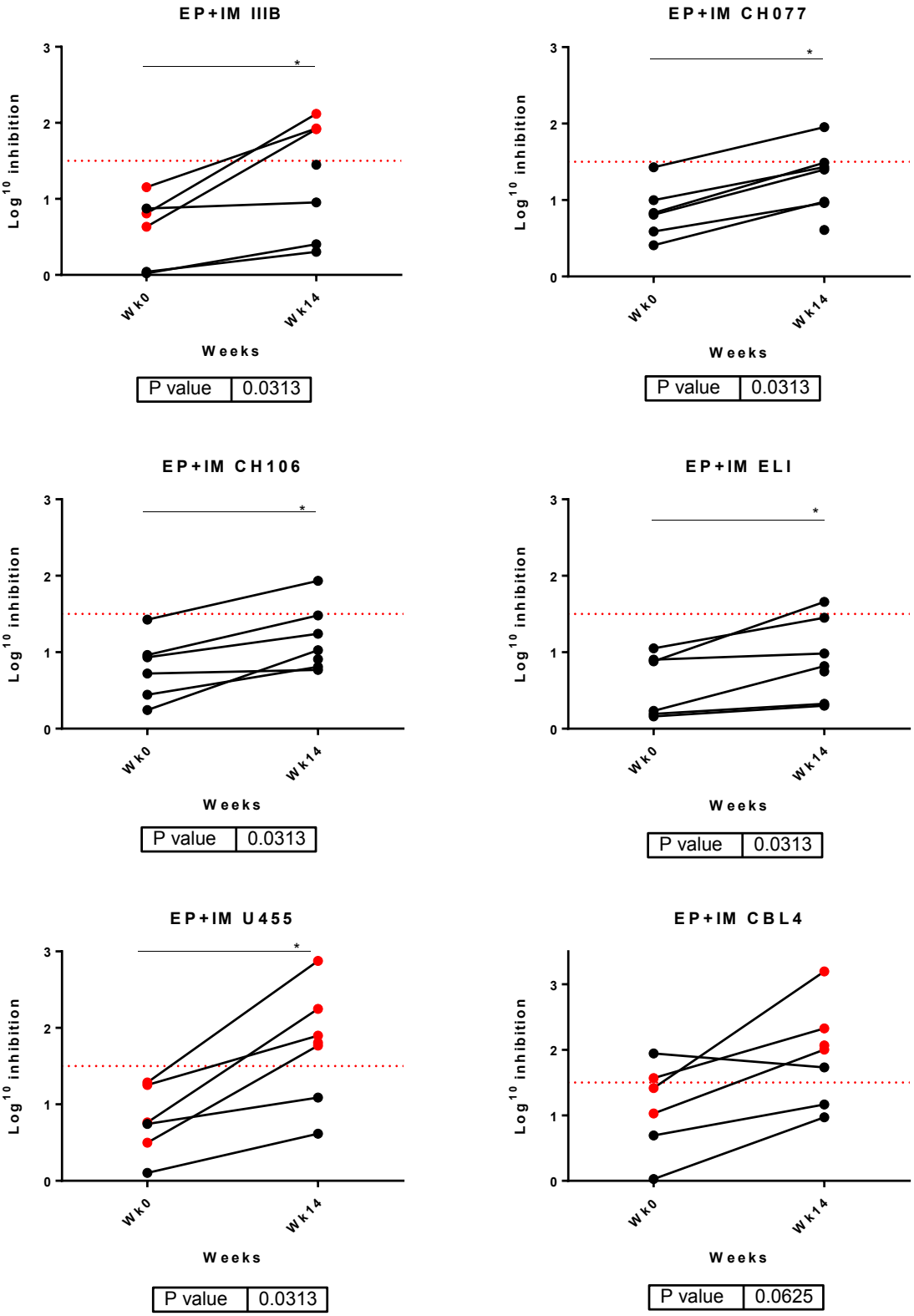


Figure 4.35: Before-after plot for EP+IM group for each individual virus showing week 0 and week 14 log^{10} inhibition, where each dot joined by a straight line is one participant's week 0 and week 14 values. The 1.5 log^{10} cut-off as shown by the red dotted line. The positive responders are shown in red. Statistical significance was set at p=<0.05.

4.5.3 Correlation of VIA with IFN-ɣ T cell ELISpots showed a weak correlation in the TC+IM group only

To assess whether there was a relationship between the IFN-ɣ T cell ELISpots and the VIA data, a correlation analysis was conducted showing no relationship between these 2 assays in the ID+IM nor EP+IM groups. In the TC+IM group there was a weak significant correlation (P=0.0386), but the R^2 correlation coefficient was not convincing at 0.08611 (Figure 4.36).

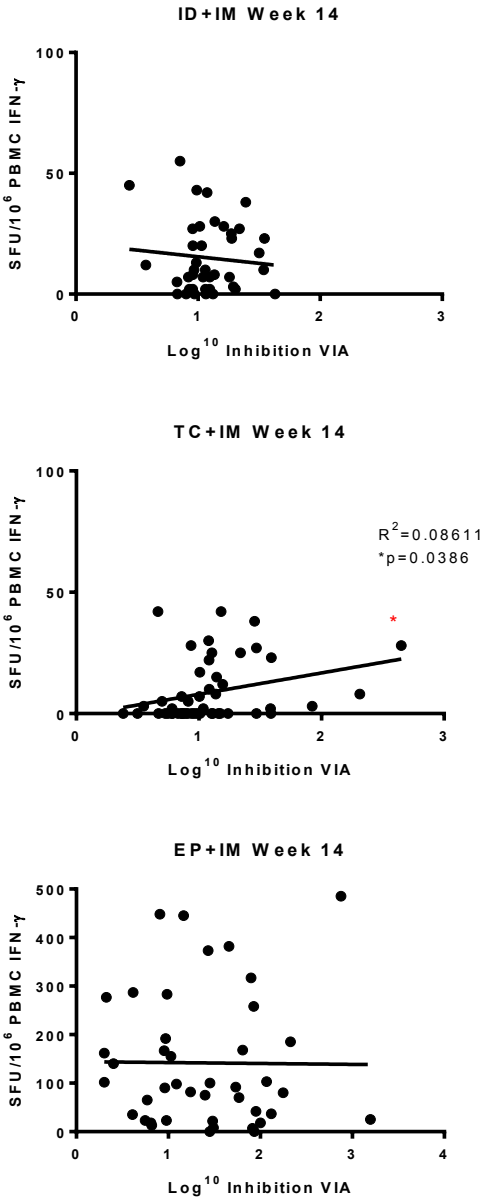


Figure 4.36: Correlation analysis with line of best fit showing correlation between IFN-ɣ T cell ELISpot data from week 14 on the y axis and log^{10} viral inhibition at week 14 on the x axis. Note the scale for the EP+IM graph is different to incorporate higher values from both assays.

Summary of Immunogenicity results: **VIA**

- The VIA showed participants in the EP+IM group inhibited a greater breadth of viruses compared to other groups
- There were a greater number of responders to the non Clade B viruses at the primary end point across all three groups
- Although the TC+IM group had no responders at the primary end point by IFN-ɣ, there were 4/9 participants inhibiting at least 1 virus
- A weak positive correlation was seen between the T cell ELISpot and VIA results in the TC+IM group only

4.6 DISCUSSION

In the first section of this chapter I aimed to determine the immunogenicity of the GTU®MultiHIV B clade DNA vaccine using 3 routes of immunisation in HIV uninfected volunteers participating in the CUTHIVAC 001 randomised phase I clinical trial, and to determine whether the T cell response is influenced by the route of vaccination.

The EP+IM group showed the greatest magnitude, breadth and duration of the IFN-ɣ response across all time points. This is perhaps not entirely surprising, as electroporation addresses the key limitations of DNA vaccination, producing a transient cell membrane permeability to substances such as DNA, and a level of inflammation in muscle tissue aiding recruitment of APCs to the vaccine site [226]. This technique of improving DNA delivery is already being utilised in cancer therapies including renal and hepatocellular malignancies [227], [228].

The magnitude of the IFN-ɣ response from my data is comparable to the first HIV DNA trial using EP which is outlined in detail in my introduction chapter (Section 1.9.1). In this placebo controlled randomised trial in HIV uninfected volunteers, IM+EP administration of the ADVAX Clade C/B DNA vaccine at 3 difference doses was investigated to assess whether EP could enhance immunogenicity compared to standard IM injection [182]. Although this is clearly a different vaccine construct to the GTU®MultiHIV B clade DNA vaccine, the mean and median IFN-ɣ magnitude (expressed as SFU/M PBMC) in *Nef* and *Gag* peptide pools from my data are greater in magnitude than those stated in the paper at the highest dose of EP+IM (4mg), and more comparable to the IFN-ɣ response in the *Env* peptide pool. ICS responses in the Vasan et al paper were also predominantly CD4+ driven as seen in CUTHIVAC 001.

In contrast, in the Swedish trial of HIV negative volunteers vaccinated with and HIV DNA vaccine using intradermal EP [183] (also detailed in Section 1.9.1), the magnitude of the IFN-ɣ response is far greater than that seen in my data at the primary end point. However, in that trial DNA was used as a primer with participants randomised to also receive MVA vaccination, therefore results may not be directly comparable.

Perhaps more surprising, is the relative paucity of the IFN-ɣ response in the ID+IM group from my data. In previous clinical trials intradermal vaccination has been shown to be immunogenic, dose sparing and highly effective particularly in hepatitis B vaccine trials and influenza [229], [188]. The trials mentioned however, did not use DNA which could be a factor, and current clinical trials in progress using intradermal DNA vaccination may produce more robust results for this vaccination route. Interestingly in the Swedish trial cited above IFN-ɣ responses in the group of participants receiving ID

DNA vaccination only (without EP), also showed very limited immunogenicity with only 1/7 responders to any peptide pool after three DNA vaccines. Further clinical trial data is pending, but perhaps early data suggests the use of DNA ID vaccination alone may not be enough trigger adequate T cell responses.

To the best of my knowledge this was the first trial using the transcutaneous route of vaccine delivery for both DNA and HIV-1 vaccination. Previous data using the TC method has examined both antibody and cellular responses using the influenza/tetanus vaccination and comparing TC administration with IM [191]. This previous study showed the TC route generated robust influenza-specific CD8+ cellular responses. With this in mind, I would have expected some T cell responses at the primary end point in this group, however none were seen by IFN-ɣ although there were some variable responses at the interim time points.

As shown from my data, assessment by conventional IFN-ɣ ELISPOTs demonstrated EP+IM DNA vaccination to be superior to other groups in terms of magnitude, breadth and duration of response. However, wider phenotypic assessment by ICS revealed a different response pattern to several cytokines, which could not have been predicted by IFN-ɣ alone. From the CD4+ and CD8+ data T cell responses were evident in all 3 groups, with a trend for greater responses in the TC+IM and EP+IM group compared to ID+IM. This is surprising for the TC+IM group when compared to lack of IFN-ɣ response at the primary end point. This may indicate targeting specialised APCs in the skin such as Langerhans cells and dermal dendritic cells can improve the quality of the response, which may be overlooked if just one cytokine is focused on. The data is also notable given the low dose of DNA applied transcutaneously and the absence of EP in this group. The utility of this route with DNA will need further characterisation.

In section B of this chapter I addressed my second hypothesis that DNA vaccination induces HIV-specific immunity that confers protection in a viral challenge *in vitro* system and aimed to determine functionality of HIV-specific T-cell responses using a VIA.

Functional assessment by VIA demonstrated vaccination using electroporation induced the most potent CD8+ viral inhibition in 71% of participants, with up to a 2.88 $\log^{10}$ reduction in viral replication. This is notable considering CD8+ responses by ICS appeared modest. The frequency of responders in the EP+IM group using this assay was similar to those observed in a previous study delivering an HIV-DNA vaccine (HIVMAG) using the same EP device [230]. In this study HIV uninfected participants were randomised to receive HIVMAG DNA alone or co-administered with IL-12 delivered intramuscularly with EP with different prime-boost regimens using recombinant Ad35. Although as

expected those receiving boosted regimens had far greater maximum log^{10} inhibition, the inhibition in groups receiving DNA alone are comparable to responses observed in my study.

Analysis of the VIA has also provided some interesting yet discordant results in comparison with the T cell ELISpots. The greatest discrepancy can be seen in the TC+IM group, with 4/9 participants displaying inhibition at week 14 compared to no responders in this group at the same time point in the ELISpot assay. Perhaps the ability of the TC route to preferentially amplify the CD8+ response is key here, although the mechanism to explain this discordance need to be further characterised [191]. Nevertheless, more emphasis is increasingly being placed on the function and quality of the T cell response, rather than focusing on phenotypic single parameter responses (such as IFN-ɣ), again raising the question of whether future vaccine trials will need to incorporate a combination of assays to address these parameters [231].

The VIA has also demonstrated cross clade inhibition of clade A, D and mixed A/D viruses, with the greatest number of participants inhibiting clade D virus. In a previous clinical trial the GTU®MultiHIV B clade DNA vaccine administered to Clade C infected subjects showed a 0.48 log drop in HIV RNA (viral load), suggesting this vaccine may have cross clade cover other than clade B, although it is difficult to draw direct comparisons as the VIA was not conducted in that specific trial [135]. One way of looking into this further could be to examine the sequence in the DNA vaccine construct and align this against viral sequences in the VIA. This was beyond the limits of my research project but could be an interesting avenue to explore in this trial.

In terms of general limitations related to the assays used, as already stated the T cell ELISpot assay assesses a single cytokine and although IFN-ɣ has been extensively used in clinical trials, taking the IFN-ɣ results alone without the ICS data, the EP+IM group would alone have shown superior results. Incorporating the ICS data shows the importance of more detailed phenotypic and functional analysis. Whilst the debate over which assay provides an adequate marker of T cell immunogenicity, both have been shown here to have individual merits that warrant their current use in monitoring T cell immune responses in HIV vaccine trials.

Limitations of the VIA include risk of contamination due the length of the assay (3 weeks in total), which did occur with a number of the participant samples. Furthermore the 6 viral strains used for this assay were chosen based on availability at the time, and not necessarily practical application. Finally with respect to the discordance between the VIA and the ELISpot results although there was consistency across the EP+IM group, there was little correlation with IFN-ɣ or ICS results. The reasons for this are unclear but suggest IFN-ɣ may be a poor surrogate CD8+ killing.

Trial-related limitations include whether lack of a standardized intradermal device for ID vaccination may have affected the results. There are now several commercial devices available for intradermal vaccination to ensure consistency of targeting the ID skin layer, and although I am confident we did achieve this with each vaccination, there is always a small risk of accidental subcutaneous vaccine delivery when conducted using conventional needle and syringe [232].

Additional limitations include whether using frozen rather than fresh PBMC samples to conduct the ELISpots with may have affected immunogenicity, and finally with such small participant numbers it is important not to overestimate the significance of the immunogenicity results.

In summary, the EP+IM group performed better by IFN-ɣ with respect to magnitude, breadth and duration of response. However, the quality and function of the immune response as assessed by ICS and the VIA has revealed discordant results particularly in the TC+IM group, the importance of which is yet to be determined. In general, it can be said that the route of vaccination does have an impact on the T cell response, with electroporation remaining a highly effective way of improving DNA uptake in this trial. In addition DNA vaccination induces HIV-specific immunity that confers protection in a viral challenge *in vitro* system in HIV negative volunteers, apparent across all three groups.

In the next 2 results chapters I address the second half of my hypothesis and aims focusing on an HIV positive cohort in a therapeutic HIV-DNA vaccine trial.

OVERALL SUMMARY OF IMMUNOGENICITY RESULTS FROM CUTHIVAC 001

- The EP+IM group showed a greater magnitude, breadth and duration of response across all peptides at the primary end point and interim time points by IFN-ɣ T cell ELISpots
- The ICS data shows a different pattern in response to several cytokines, which could not have been predicted by IFN-ɣ alone particularly in the TC+IM group with CD4+ and CD8+ data comparable to EP+IM
- The VIA showed participants in the EP+IM group inhibited a greater breadth of viruses compared to other groups with the maximum log^{10} viral inhibition
- Although the TC+IM group had no responders at the primary end point by IFN-ɣ, there were 4/9 participants inhibiting at least 1 virus
- The overall discordance found between T cell ELISpots and the VIA in this trial highlights the importance of assessing all aspects of the immune response including quality and function
- Electroporation remains a highly effective method of improving DNA uptake into cells and improving DNA immunogenicity, addressing my hypothesis that HIV-specific immunity induced by DNA is influenced by the route of vaccination in HIV negative volunteers
- In this trial DNA vaccination across all three groups but particularly in the EP+IM group induced HIV-specific immunity that conferred protection in a viral challenge *in vitro* system in HIV negative volunteers addressing my second hypothesis

Chapter 5
Results: Trial Data CUTHIVTHER 001

5.1 INTRODUCTION

As the fourth decade of the HIV epidemic advances, the progress and development of safe and effective ART has led to almost near normal life expectancy in people living with HIV, if diagnosed early and established on treatment [233]. However, ART whilst able to control infection cannot eradicate it, as evidenced by viral rebound after treatment interruption [234]. In addition whilst highly effective in controlling viral replication, ART is expensive and global access remains a major public health issue.

The rationale for the development of a therapeutic vaccination is based on several immunological key factors. These include the inability of natural infection with HIV to elicit sufficient immunity to control replication, HIV infection itself causing major abnormalities in the cellular immune responses and as mentioned the failure of ART to eradicate established infection [136]. To date no therapeutic vaccine candidate has been show to prevent viral rebound following treatment interruption. However, current and future clinical trials are providing increasing evidence for the role of therapeutic immunisation in combination with other, newer immunological approaches to establish a functional cure in those already living with HIV on established antiretroviral therapy [235].

CUTHIVTHER 001 was designed to explore the safety and immunogenicity of 2 different delivery modes of the GTU® MultiHIV B clade DNA vaccine in HIV positive volunteers on ART in comparison to placebo. This was a Phase I/II trial based on encouraging safety data from the first trial. In addition, data from the Fit-06 trial in South African Clade C infected volunteers who had never received ART showed in both IM and ID delivery the vaccine was safe and well tolerated with no vaccine related serious adverse events [135]. As the vaccine was based on a clade B sequence, trial inclusion was limited to Clade B infected participants only, with the aim of maximising the chances of a successful outcome.

Amongst people living with HIV, the vast majority have detectable HIV-specific immune responses primed by autologous viral infection without vaccination. The main differences between this trial and CUTHIVAC 001 was in this study the participants were all HIV positive on successful ART as evidenced by undetectable HIV viraemia. The design of this study differed by the addition of a placebo group in anticipation of varying and potentially high background measurable HIV-specific

immune responses to peptides in HIV positive volunteers, and the comparison of the TC+IM and EP+IM groups only.

In this trial the ID+IM route of delivery was omitted based on the CUTHIVAC 001 trial showing only limited responses (amongst HIV uninfected individuals) in this group. Although the TC+IM group also showed limited responses by IFN-ɣ it was felt the potential for primed CD8+ responses in HIV positive individuals might produce interesting results and this would be the first time TC DNA vaccination in combination with IM would be investigated in an HIV positive cohort. A final major difference was the dose of IM vaccination was reduced from 4mg (2 IM doses) to 2mg (1 IM dose). This was predominantly because of the 1 participant in the preventative trial who did not want to continue after the first EP procedure, therefore a decision by the trial committee was made to electroporate just one leg and therefore deliver a single IM dose. It was expected this lower dose would still be adequately immunogenic amongst HIV positive participants who likely already have some CD8+ T cell priming.

As with the previous trial, the primary safety endpoint was defined as the presence of a grade 3 or above local or systemic solicited adverse event or an adverse event that resulted in a clinical decision to discontinue further immunisations. All adverse events were graded according to the FDA toxicity grading scale.

Data on safety events were recorded from all visits as well as from participant diary cards. Based on the overall safety at the day 1 safety visit in the CUTHIVAC trial, a decision was made by the trial team and incorporated into the protocol to exchange the day 1 safety visit for a telephone call instead. The remainder of the visits were the same as in CUTHIVAC 001 (Figure 5.1).

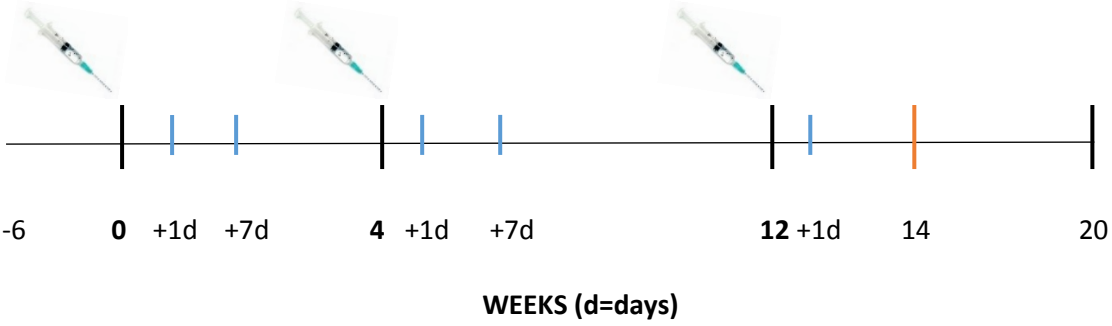


Figure 5.1: Vaccination schedule CUTHIVTHER 001. Diagram to show the 3 vaccination time points at weeks 0, 4 and 12. The primary end point is shown in orange at week 14, and week -6 denotes the 6 week period between screening and possible enrolment. After each vaccination there was a safety phone call at Day 1 and a safety visit at Day 7 as indicated and the final follow up was at week 20. Both groups followed the same vaccination schedule.

As mentioned, this was the first time TC DNA vaccination was investigated in an HIV positive cohort and in combination with IM injection. In addition the use of EP assisted DNA delivery in an HIV positive cohort is also novel, although one trial has recently completed publication using a different DNA vaccine delivered EP+IM [184]. Therefore once again the documentation and presentation of the safety data is important to guide future vaccine studies.

5.2 RESULTS

5.2.1 Participant Recruitment

Participants were initially recruited for this trial from St Mary's hospital HIV cohort. With the help of the research team all Clade B infected patients were identified. Next Clade B participants were further selected to those with the appropriate CD4 counts (nadir >250 cells/mm^3), viral loads (<50 copies/ml on 2 separate occasions in the last 6 months) and negative hepatitis B (HepBsAg negative) and C status (PCR negative) as per inclusion/exclusion criteria. The rationale for this CD4+ cut-off and an undetectable viral load was to aim to recruit a heathy cohort with relatively preserved immune function despite HIV infection. The clinicians of potentially eligible participants were then contacted by email to ensure they were happy for us to contact their patients. This was then done by phone, email, or when the patient next came into clinic. We also had direct referrals from clinicians.

There was considerable time pressure to recruit 30 participants for this trial, as the vaccine expiry date was fast approaching, therefore recruitment was opened up to other HIV clinics within Imperial College NHS Healthcare trust following approval by ethics on this protocol amendment. The cumulative accrual of participants to the trial is shown in the graph below (Figure 5.2).

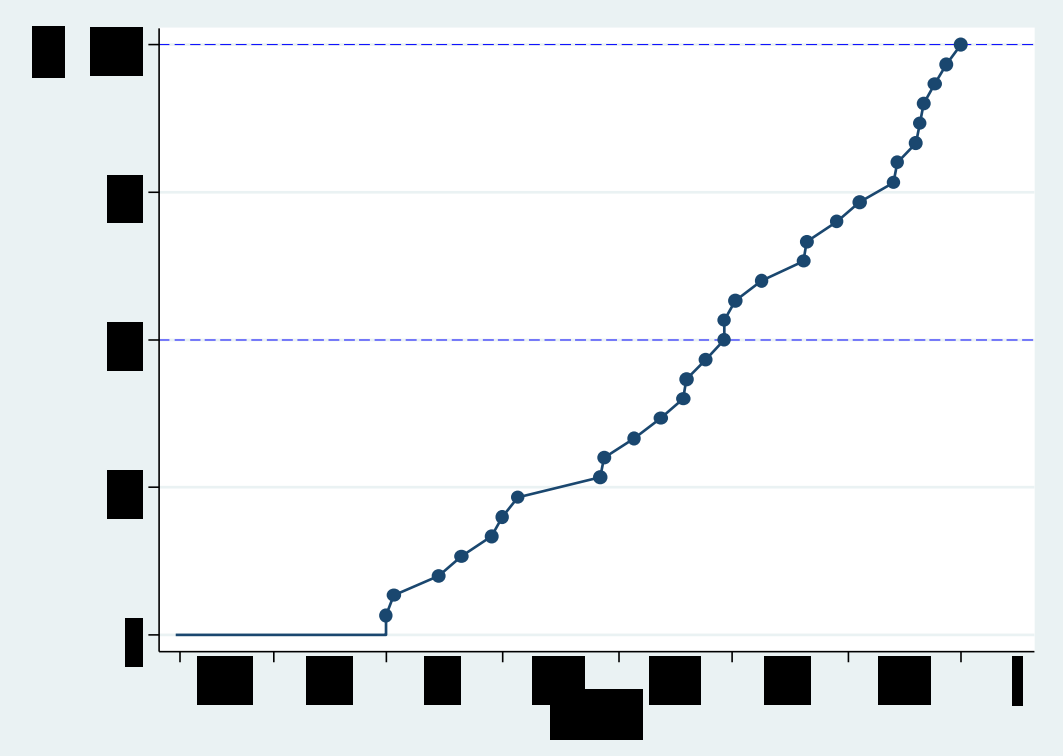


Figure 5.2: Graph to show cumulative accrual of participants to the trial by time.

5.2.2 Enrolment and participant flow

Enrolment occurred between June and November 2015. In total I screened 36 participants to enrol the target of 30 into the trial (screening to enrolment ratio 1.2:1). Although recruitment may have taken a similar length of time to the previous trial (approximately 6 months), lessons from experiences in recruitment to CUTHIVAC 001 led to a more streamlined pathway for this study. This included access to serial blood tests on the patients from previous routine HIV outpatient appointments (including liver function tests) and clinical review of notes to help identify potential exclusion factors. This resulted in the screening of far fewer people as greater effort was placed on pre-screening, which improved the odds of participants being eligible once screened.

From the 6 participants who did not progress into the trial after screening, 2 were eligible but withdrew consent due to work commitments, 1 had a skin condition which may have interfered with transcutaneous vaccination (keratosis pilaris), 1 had a BMI lower than the inclusion/exclusion criteria, and 2 had abnormal liver function tests above grade 2 (hyperbilirubinaemia, and raised ALT).

All participants enrolled were male, with a median age of 34 years (range 21-43 years), of White ethnic origin (90%), and most were in full time employment (83%) (Table 5.1). Female sex was not a

trial exclusion criteria, but anecdotally the few women who did show an interest were planning on starting families or were Clade C and therefore not eligible. 57% of participants reported recreational drug use in the preceding last 6 months. In total 15 participants were randomised to each group, with a further randomisation to receive active drug (GTU®MultiHIV B clade DNA) or placebo (saline).

Table 5.1: Participant demographics (all participants enrolled)

Demographics	All (n=30)
Sex	
Male	30 (100%)
Age at screen (years)	
Median	34
IQR	30-37
Range	21-43
Ethnic Group	
White British	10 (33%)
White Other	17 (57%)
Other ethnic group	3 (10%)
Employment	
Full time	25 (83%
Currently smoking	12 (40%)
Alcohol use (units)	
Median	6
IQR	2-10
Range	1-25
Recreational drug use in last 6/12	17 (57%)

In total, 1 participant in the EP+IM group (active arm) did not complete the vaccination schedule but remained in follow up. CT017 received 2 vaccination doses as per protocol. However at his week 5 safety visit (1 week following second vaccine), where he was assessed to be well with no new adverse events and no new medications, safety bloods were taken in accordance with the protocol which showed his ALT had more than doubled (from 23 to 54), his AST had also risen (from 29 to 47) and his HIV viral load had increased from <20 to 59 (although this is not classed as a clinically important 'blip' in viral load).

I recalled the participant for further assessment where I took a full history including sexual history and drug history (plus recreational drugs) and conducted a full liver screen. His physical examination was unremarkable. Following these blood tests his hepatitis C (HCV) PCR was found to be positive (antibody negative) with a HCV VL of >18million copies/ml indicating a diagnosis of acute hepatitis C.

This diagnosis was unrelated to the trial vaccination procedure. I referred him to the co-infection clinic for management and follow up and he subsequently did not receive the final vaccine for safety reasons but attended all follow up visits.

All other participants completed the full vaccination schedule (Figure 5.3: Consort Diagram).

In terms of participant motivation for volunteering, 28 participants (93%) were interested in helping and advancing HIV research, with 4 participants specifically mentioning HIV cure research as their main motivation. There were no participants who volunteered for purely financial reasons (Figure 5.4) despite receiving financial compensation (a total of £1100) for this trial. This again was a pre-defined and agreed compensation amount with the ethics committee responsible for this trial.

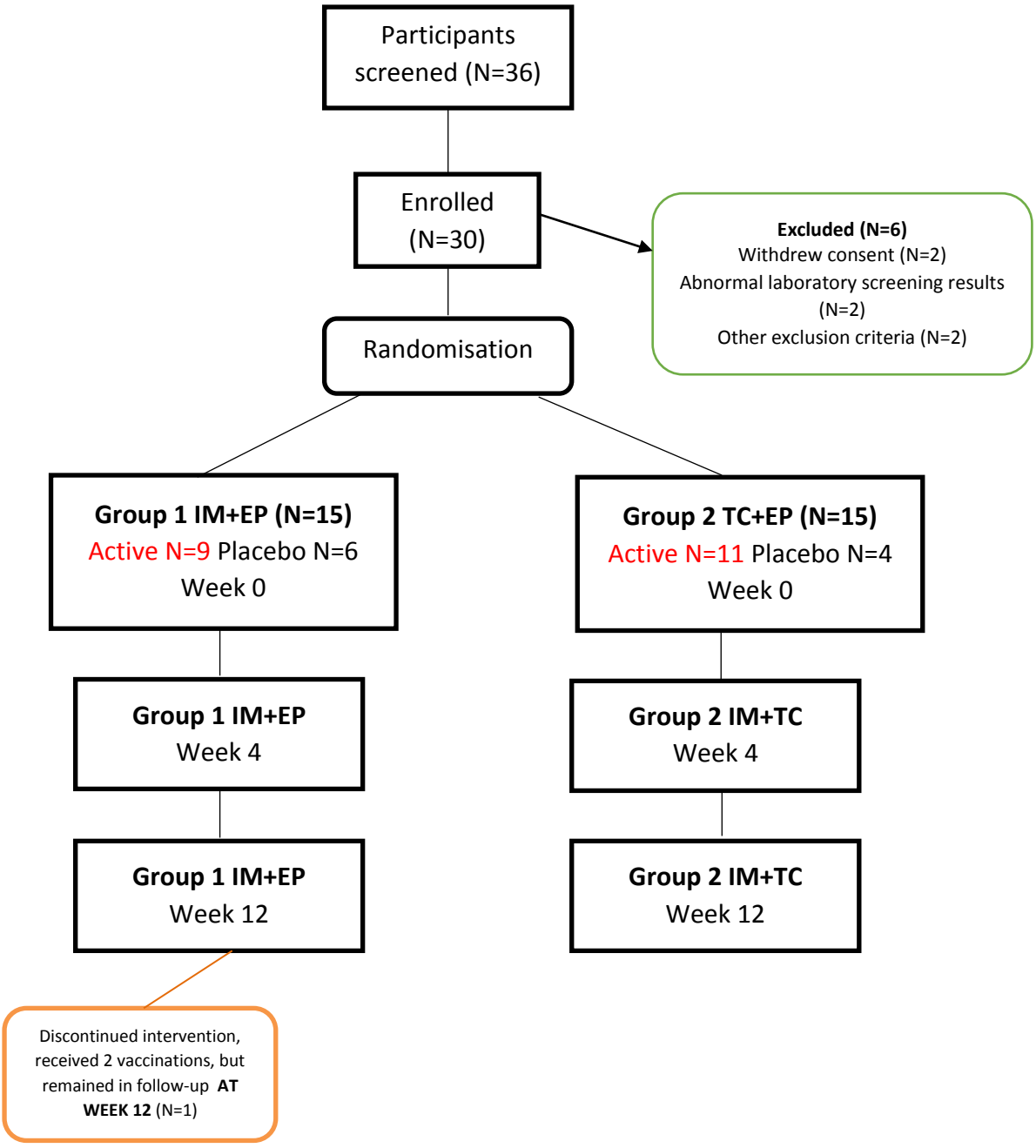


Figure 5.3: Consort diagram outlining participant flow in the 2 groups.

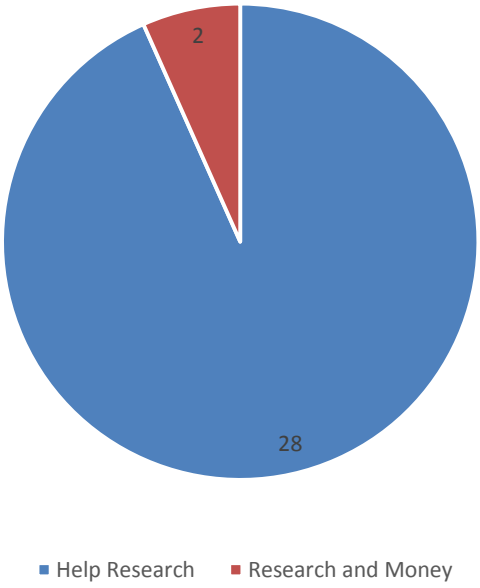


Figure 5.4: Participant motivation for taking part in the CUTHIVTHER 001 trial (N=30)

5.2.3 HIV related characteristics

29/30 participants identified as MSM with one participant identifying as bisexual. The median number of years since diagnosis of HIV was 3 years in this cohort (range 0.9 to 11.5 years), and participants had relatively high nadir CD4 counts (median 346; range 260-968 cells/mm^3). To be enrolled in the study all participants had to be on ART for at least 6 months, and the mean number of years on ART was 2.18 years with a combination of regimens, the most common of which was triple therapy with an NRTI plus NNRTI (Table 5.2).

Table 5.2: HIV related characteristics (all participants enrolled)

HIV related characteristics	All (n=30)
Sexuality	
MSM	29 (97%)
Bisexual	1 (3%)
Regular partner	17 (57%)
Years since HIV diagnosis	
Median	3
IQR	1.6-7
Range	0.9-11.5
CD4 nadir (cells/mm^3)	
Median	346
IQR	300-516
Range	260-968
Last VL <50 copies/ml	30 (100%)
Time on current ART regimen (years)	
Mean	2.18
IQR	1.14-2.90
Range	0.6-7.18
ART regimen at enrolment	
NRTI + NNRTI	20 (67%)
NRTI + PI	5 (17%)
NRTI + INI	4 (13%)
Other	1 (3%)

5.2.4 Safety and reactogenicity

In this trial the GTU®MultiHIV B clade DNA vaccine was safe and well tolerated across the active groups compared to placebo and there were no serious adverse events experienced throughout the trial (as measured by the FDA toxicity grading tables).

The summary of solicited events from participant diary cards, the immunisation form (conducted in clinic 60 minutes post vaccination), and the safety phone call (at day 1) shows there were no significant differences in systemic symptoms between the EP and TC groups receiving active drug (p=0.98) (Table 5.3 and Figure 5.5). Surprisingly, in the EP (placebo) group, there were still a proportionally high number of systemic symptoms reported, suggesting even without DNA there may be physical side effects from electroporation.

In terms of local arm reactions, there were a greater total number of events in the TC (active) group compared to placebo (53 events compared with 18 events respectively), but this did not reach statistical significance (p=0.62). With respect to local leg reactions, there were no significant differences in the EP (active) group in comparison to EP (placebo) or conventional IM injection in the TC (active) group, despite a greater number of events recorded. All events were graded as 1 or 2 and there were no grade 3 or above events.

For the unsolicited events, the majority were either possibly or unlikely to be related to vaccination. This relationship to study drug was determined by the trial team. All unsolicited events were also grade I or 2 (FDA toxicity grading tables) with no higher grades reported (Table 5.4).

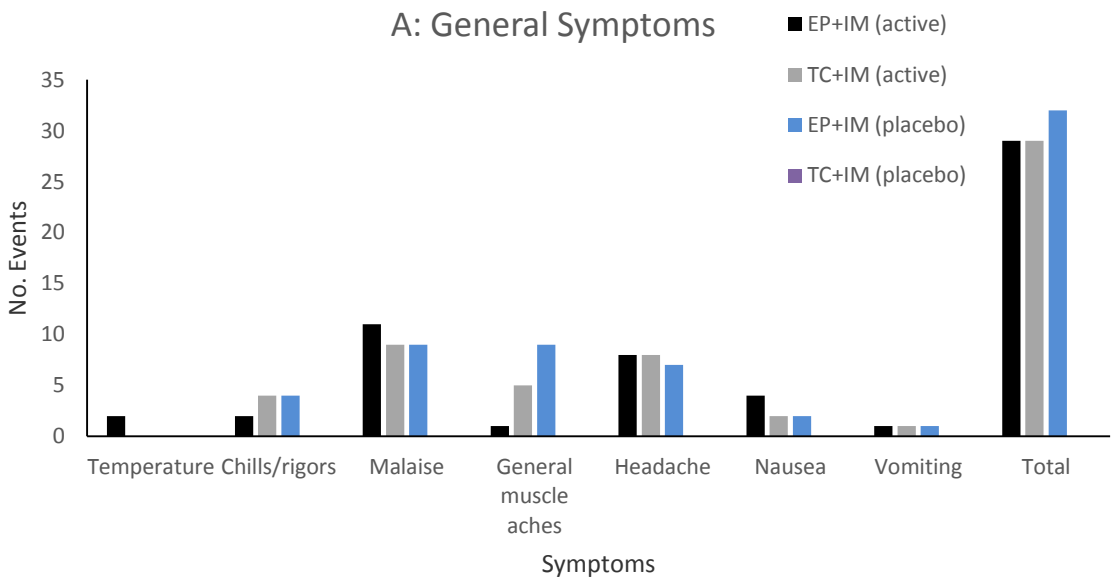
In this study 7 participants developed hyper or hypo pigmentation in the TC+IM (active) group and 1 in the TC+IM (placebo) group. This was perhaps to be expected considering my experience from the previous trial, and all had resolved by the end of the trial. Perhaps unexpectedly, there was also a participant receiving placebo who experienced skin changes. I contacted the dermatology lead who developed this technique in Berlin, (Dr Annika Vogt at Charite) to discuss this, who was also surprised as in their development of the TC technique they had not experienced pigmentation disorders using saline (unpublished data).

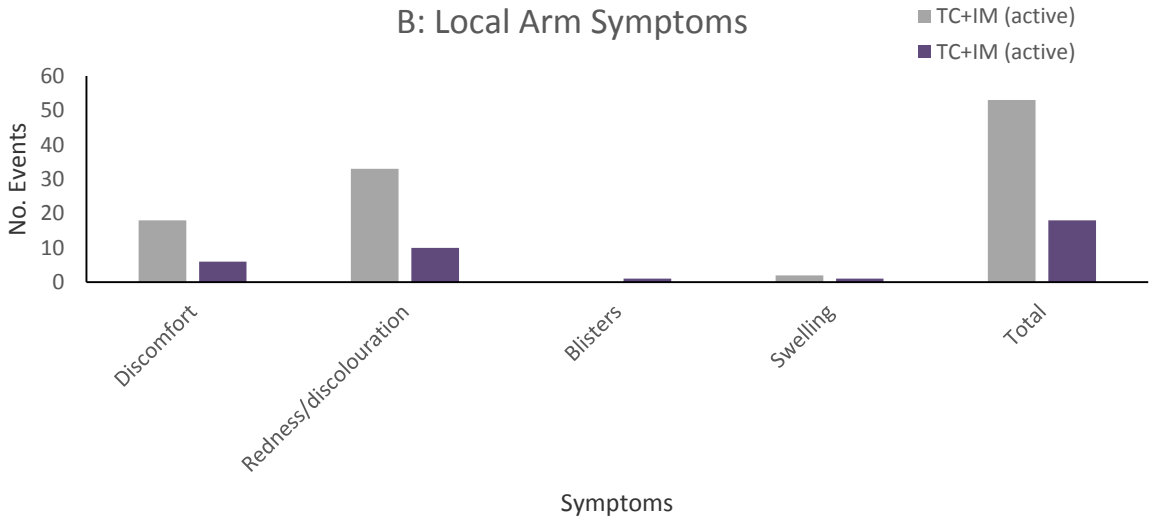
Table 5.3: Summary of solicited adverse events occurring over the entire trial by group. Data includes recorded events from immunisation form conducted in clinic at 60 minutes post vaccination, the day 1 safety phone call, and participant diary cards. If the same symptom was documented by a participant over a number of days, this was counted as 1 event. There is data for local arm symptom in the EP groups as they did not receive vaccination to the arms. For the local leg symptoms this is a comparison of EP+IM vs the conventional IM injection given into the upper thigh after TC vaccination. Statistical significance was set at p=<0.05.

	Symptoms	**EP+IM (active) (n=9)**	**EP+IM (placebo) (n=6)**	**TC+IM (active) (n=11)**	**TC+IM (placebo) (n=4)**
Systemic reactions (p=0.98 EP active vs TC active)	Temperature Chills/Rigors Malaise General muscle aches Headache Nausea Vomiting **Total**	2 2 11 1 8 4 1 **29**	0 4 9 9 7 2 1 **32**	0 4 9 5 8 2 1 **29**	0 0 0 0 0 0 0 **0**
Local reactions Arm (p=0.63 TC active vs TC placebo)	Discomfort Redness/Discolouration Blisters Swelling **Total**	n/a	n/a	18 33 0 2 **53**	6 10 1 1 **18**
Local reactions Leg (p=0.57 EP active vs EP placebo) (p=>0.99 TC active vs TC plac) (p=0.31 EP active vs TC active)	Discomfort Redness/Discolouration Blisters Swelling **Total**	22 23 0 9 **54**	17 17 0 5 **39**	3 16 0 0 **19**	1 5 0 1 **7**

Table 5.4: Summary of unsolicited adverse events by group with relationship to vaccine shown. All unsolicited adverse events were documented as grade 1 or 2.

Safety: Unsolicited Adverse events	**EP+IM (active)**	**EP+IM (placebo)**	**TC+IM (active)**	**TC+IM (placebo)**	**Total (%)**
Definitely related to vaccine	0	0	0	0	0
Possibly related	4	8	19	4	35 (43%)
Probably related	0	0	14	2	16 (20%)
Unlikely related to vaccine	10	9	6	2	27 (33%)
Not related	1	0	3	0	4 (5%)
Total	**15**	**17**	**42**	**8**	**82 (100%)**


A: General Symptoms
EP+IM (active)
TC+IM (active)
EP+IM (placebo)
TC+IM (placebo)
No. Events
35
30
25
20
15
10
5
0
Temperature
Chills/rigors
Malaise
General muscle aches
Headache
Nausea
Vomiting
Total
Symptoms


B: Local Arm Symptoms
TC+IM (active)
TC+IM (active)
No. Events
60
50
40
30
20
10
0
Discomfort
Redness/discolouration
Blisters
Swelling
Total
Symptoms

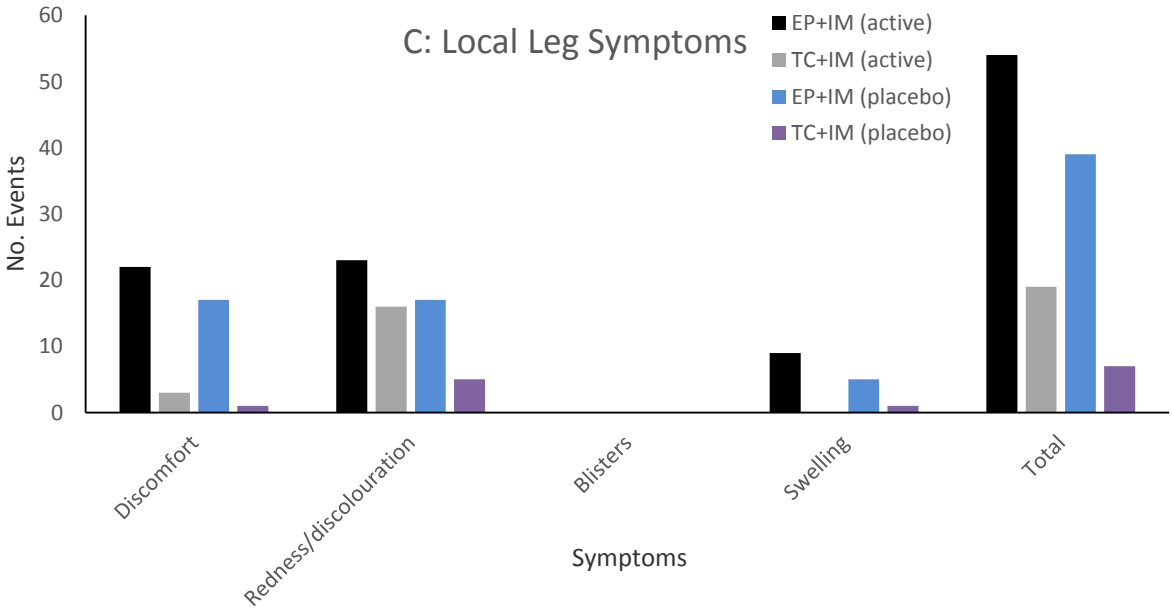


Figure 5.5 A: Bar chart to show general symptoms documented from immunisation form conducted 60 mins post vaccination, day 1 safety call and participant diary cards. B: Local arm symptoms. NB This is the TC group only as EP+IM groups did not received vaccination to the arm. C: Local leg symptoms. NB This compares EP+IM (active or placebo) to conventional IM vaccination given in the TC groups (IM injection into the thigh).

5.2.5 Tolerability and acceptability data (EP+IM group only)

As in the previous trial, participants in this group were asked to fill out pain questionnaires following each procedure and also an acceptability questionnaire. The visual analog pain score used ranged from 0 (no pain) to 10 (worst ever pain) and participants were asked to rate their pain immediately after EP and 30 minutes later.

The EP tolerability data shows little change in mean or median pain score between placebo and active groups either immediately after EP or 30 minutes later (Table 5.5), suggesting no difference in pain scores in participants receiving active drug or placebo and the pain documented was likely due to the EP procedure itself. Reassuringly the pain did decrease to minimal levels after 30 minutes in both groups. 100% of participants indicated EP was acceptable if it protected people from a serious disease for which no vaccine was currently available such as HIV.

Table 5.5: Tolerability data for EP+IM active and placebo groups showing mean and median pain scores with interquartile range immediately after EP and 30 minutes later for each vaccination.

	EP+IM (active) (n=9)	**EP+IM (placebo) (n=6)**	**EP+IM (combined) (n=15)**
Pain at vaccination 1			
Immediately after EP			
Mean	5	5.8	5.3
Median (IQR)	4 (4-6)	6 (5-6)	5 (4-6)
30 mins after EP			
Mean	1.2	2.5	1.7
Median (IQR)	1 (1-2)	2.5 (2-3)	2 (1-3)
Pain at vaccination 2			
Immediately after EP			
Mean	4.5	5.7	5
Median (IQR)	5 (4-6)	6 (5-6)	6 (4-6)
30 mins after EP			
Mean	1.9	1.5	1.7
Median (IQR)	1 (1-3)	1.5 (0-2)	1 (0-3)
Pain at vaccination 3			
Immediately after EP			
Mean	4.4	5	4.6
Median (IQR)	3.5 (3-6.5)	6 (5-6)	4 (3-6)
30 mins after EP			
Mean	0.5	0.7	0.6
Median (IQR)	0 (0-1)	0.5 (0-1)	0 (0-1)

5.3 DISCUSSION

In this chapter I aimed to determine the safety of the GTU®MultiHIV B clade DNA vaccine using 2 routes of immunisation in an HIV positive cohort participating in the CUTHIVTHER 001 randomised phase I/II clinical trial, in comparison to placebo.

Recruitment in this trial was also a challenge, but valuable experience had been gained from the previous trial and one important advantage was access to serial blood tests in this cohort, which helped reduce the number of exclusions at screening. In terms of demographics of those enrolled into the trial this was a relatively young male cohort. Although the trial was open to male and female participants, the participants enrolled reflects the cohort at St Mary's hospital. In addition, historically women are less likely to enrol into clinical trials particularly HIV clinical trials for a multitude of reasons including pregnancy or wanting to conceive [236], and many HIV positive women in the St Mary's cohort were not clade B infected.

In this trial 93% of volunteers documented the main motivation for participating was to help advance HIV research, with 4 of these participants mentioning a specific interest in HIV cure research. This is in comparison to only 31% of participants in the CUTHIVAC trial of HIV uninfected volunteers. This is perhaps not surprising considering participants in this trial were already living with HIV and likely invested in the disease and future course of the HIV epidemic. Despite this highly motivated cohort of participants, the consent process for trial participation had to remain robust and follow all ethical guidelines as in other clinical trials, with a personal effort on my part as the trial physician to make it very clear this was an early study and specifically not designed as a 'cure' study. Phrases such as 'cure' may have led to unrealistic expectations or therapeutic misconceptions about the benefits of participation, crossing ethical boundaries.

This cohort had a median of 3 years since diagnosis and high nadir CD4 counts (median=346 cells/mm^3), indicating a relatively healthy cohort with potentially low immune dysfunction. In addition 10/30 participants were referred to our team directly from the 'HIV Reservoir targeting with Early Antiretroviral therapy' or HEATHER observational study. This observational cohort of over 250 individuals who have initiated ART in primary HIV (defined as within 6 months of a previous negative HIV test) have been shown to have smaller HIV reservoirs quantified by HIV-1 DNA and enhanced recovery of CD4/CD8 ratio as a direct result of early treatment [237]. The impact of including these individuals on the immunogenicity results from this trial is discussed in the next chapter.

Other notable details from the demographics data are that 57% of participants in this study had reportedly taken recreational drugs in the past 6 months, a figure that may seem disproportionately

high. However, recent data from the cross sectional ASTRA study which examined recreational drug use in HIV positive MSM across 8 UK HIV clinics has reported similar rates of 51% [238]. To date there is little data to determine the link between recreational drug use and potential attenuation of the immune response, although the effect of alcohol and smoking have both been previously well documented [239].

In addition, sexualised substance use termed 'chemsex' has been linked to several sexually transmitted infections, including hepatitis C in MSM [240]. The prevalence of hepatitis C infection in those with HIV in the UK is now estimated to be 8.9%, with the highest risk in those who inject drugs, or in MSM in large urban centres (including London) where sexual transmission is the predominating risk factor [241]. In this trial one person became infected with hepatitis C linked to sexual risk and chemsex (but unrelated to the vaccine).

With respect to patient safety, there were no serious adverse events in this trial with all solicited and unsolicited adverse events a maximum grade of 2. The safety data does indicate there were systemic symptoms in the EP+IM placebo group which may be as a result of the actual procedure, although there were more local leg reactions in the EP+IM active group.

As expected from the previous trial, pigmentation changes occurred in the TC+IM group (mostly in those receiving active drug). This was less of a surprise due to my previous experience from CUTHIVAC, the proposed mechanisms for which I have outlined in Chapter 3. No participants declined further vaccination as a result of hypo or hyperpigmentation skin changes, potentially due to better pre-procedure counselling by the team, and knowing all the changes had resolved in the previous trial enabled me to reassure participants experiencing hypo or hyperpigmentation that is was highly likely all changes would resolve. In addition, there was no evidence to suggest the TC procedure was better tolerated with less local or systemic side effects comparing the HIV negative to HIV positive cohort in both trials. This has also been shown in a previous trial using the TC procedure and a Tetragrip vaccine (not DNA) comparing immune responses in HIV uninfected and HIV positive volunteers [191].

The pain scores from participants in the EP+IM group showed pain improved after 30 minutes as expected with the EP procedure and seen in the previous trial. There were minimal differences in pain scores between the vaccine and placebo arms, and this has also been shown in a recent trial using EP+IM in an HIV positive cohort [184]. A comparison of pain scores between this trial and the previous trial in HIV uninfected volunteers (CUTHIVAC 001, Chapter 3) showed very little difference in median pain score between the two cohorts, despite this cohort of positive volunteers receiving EP to only one leg compared to both legs in the previous trial.

It is important to point out in this trial the decision to conduct EP in one leg only, was based on the first participant in the previous trial choosing to discontinue with EP secondary to pain. In hindsight, this event did not re-occur in either trial and it may have been premature to alter the protocol (and DNA dose) based on 1 participant's experience. Whether this may have also affected immune responses is discussed in the next chapter.

Limitations are similar to the previous trial including small participant numbers in each group, and lack of skin biopsies or changes in inflammatory markers recorded in those showing pigmentation changes related to the TC procedure. Another limitation is that I could only enrol patients from within Imperial College NHS Healthcare trust and therefore could not utilise other resources such as HIV related patient groups and other useful resources for recruitment.

In summary, the vaccine was safe and tolerable according to pre-specified criteria in this HIV infected cohort with similar safety findings from the previous trial. There was only 1 clinician led decision to discontinue vaccination (new hepatitis C diagnosis) which was unrelated to the study or vaccine. The immunogenicity results are presented in the next chapter.

Summary of safety and tolerability data for CUTHIVTHER 001

- The GTU®MultiHIV B clade DNA vaccine was overall safe across the 2 active treatment groups with no adverse events recorded over Grade 2
- In the EP+IM placebo group there were a large number of systemic and local leg symptoms documented suggesting the actual EP procedure may be the cause of this rather than DNA
- There were no participant decisions to discontinue vaccination but 1 clinical decision to discontinue due to a new acute hepatitis C diagnosis, unrelated to the study
- There were no serious adverse events in this trial with no safety end points met
- There were no differences comparing safety data from this trial and the uninfected cohort (CUTHIVAC)

Chapter 6
Results: Immunogenicity Data CUTHIVTHER 001

SECTION A: IFN-ɣ T CELL ELISPOTS

6.1 INTRODUCTION

Hypothesis

5. DNA vaccination induces HIV-specific T cell immunity in **HIV positive** volunteers, and is influenced by the route of vaccine administration
6. DNA vaccination induces HIV-specific immunity that confers protection in a viral challenge *in vitro* system in **HIV positive** volunteers

Aims/Objectives

5. To characterise T cell responses in **HIV positive** volunteers immunised with a DNA vaccine, and to determine whether T cell responses are influenced by the route of vaccine administration
6. To determine functionality of HIV-specific T-cell responses in a novel *in vitro* viral inhibition assay amongst **HIV positive** volunteers immunised with an HIV DNA vaccine

In this trial, the same GTU®MultiHIV B clade DNA vaccine was used but in an HIV positive cohort on ART, using 2 routes of vaccination from the previous trial; EP+IM and TC+IM. There was also a placebo group in this trial as it was anticipated this cohort may have existing background immune responses to peptide pools, and therefore a placebo group was needed to compare responses with those receiving active drug. These groups will be referred to as EP+IM and TC+IM, indicating if they received the vaccine (active) or placebo.

As highlighted in the introductory chapter (Chapter 1), this DNA vaccine demonstrated encouraging results in the FIT-06 Phase II trial in South Africa, where a cohort of HIV-1 Clade C positive participants who were not yet on ART were enrolled. Participants were allocated to either vaccine or

placebo groups with IM or ID administration of the vaccine (administered without EP). Significant declines in viral load (0.5 log decrease) and increases in CD4 counts were more pronounced in the IM group and in particular HLA genotypes (predominantly B*5703), with significant increases in HIV specific CD4+ and CD8+ cells post vaccination in both groups [135]. Although the cohort in the FIT-06 trial were not on ART, and the cohort in CUTHIVTHER are on ART with controlled viraemia, some comparisons can be drawn between the two clinical trials. Another major difference in the current trial, is all the participants where Clade B infected to match the vaccine construct.

In this chapter I discuss the immunogenicity results for CUTHIVTHER 001, focusing on the second half of my hypothesis and aims in an HIV positive cohort. As in the previous chapter, I conducted all the IFN-ɣ ELISPOT assays and the VIA myself. The ICS data is presented as supporting work as I did not conduct the assay myself, but did analyse the results which are included here to show an additional evaluation of the quality of the immune response.

As described in the introductory chapter, one of the challenges of developing a therapeutic vaccine has been how best to assess vaccine efficacy. Some therapeutic vaccine trials use an analytical treatment interruption (ATI) where ART is stopped temporarily and various outcome measures are used to assess vaccine induced immune-mediated viral control [129],[130]. Other trial outcomes can include clinical end points such as changes in CD4+, CD4:CD8 ratio or the development of specific clinical events [132],[133]. ATI was not included in this trial protocol as a primary outcome measure of vaccine efficacy.

The standardization of laboratory assays to define efficacy has remained poorly described in the literature. Therefore determining vaccine efficacy in this trial was challenging. Where previously clear cut standardized definitions exist for the IFN-ɣ response in HIV preventative trials, few exist in this setting. Taking into account the likelihood of pre-existing baseline responses in this cohort, the definition of a positive response for this trial is a doubling or 2-fold increase in frequency of IFN-ɣ from baseline (week 0). Should a baseline response be absent, then the same criteria as in CUTHIVAC 001 can be applied which defines a positive response as >4 x mean background (if the background response is more than 0) and >55 background subtracted SFU/M [199],[200].

Overview of methods and time points tested

For a detailed overview of the immunogenicity sampling schedule please refer to Table 2.3 in Chapter 2. In brief, bloods samples were collected from participants at the specified time points with PBMC processing according to GcLP. Samples were stored in batches at -80˚C until analysis for

immunogenicity outputs. T cell immunogenicity was assessed by IFN-ɣ ELISpot assays on frozen PBMCs stimulated by peptide pools matched to the GTU®MultiHIV B vaccine as in the previous trial. An additional integrase peptide pool was added as a control to the assay as the presence of a peptide pool not included in the vaccine may give information on any non-specific changes occurring within T cells related to vaccination.

All the immunogenicity results presented are based on an intention to treat (ITT) analysis where samples from all time points from all participants are included. A reminder of the vaccination schedule in this trial is shown (Table 6.1).

Table 6.1: Vaccine schedule for CUTHIVTHER 001. Vaccination time points, safety visits and final visit shown. The primary end point is highlighted in red.

Visit number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Nature of visit	**Screen**	**Dosing visit**	**Safety Call**	**Safety visit**	**Dosing visit**	**Safety Call**	**Safety visit**	**Dosing visit**	**Safety Call**	**Safety visit**	**Follow-up visit**
Week	-6	0	0+1day	1	4	4+1day	5	12	12+1day	14	20
										Primary end point	

In addition to the above assays, HLA typing was conducted at the week 0 baseline visit. Genetic factors such as the HLA class I genes have been shown to contribute to the variation in viral control in HIV infected individuals. These HLA proteins present viral peptides on the surface of APCs to cytotoxic T cells and is thought to be the major mechanism by which these alleles are protective and slow HIV disease progression [242]. In particular, HLA B*27, B*57, B*58:01, B*81:01 and B*51 have been identified as protective in HIV infection and important in viraemic control [243]. Finally as described later in this chapter, functional T cell responses were assessed using a modified VIA assay.

6.2 RESULTS: IFN-ɣ T CELL ELISPOTS

6.2.1 Variability of baseline responses at Week 0 (pre-vaccination)

At the week 0 pre-vaccination time point, a number of participants in each group showed pre-vaccination responses to each peptide pool, as expected in this HIV infected cohort. In particular, 24/30 (80%) and 26/30 (87%) of participants had pre-existing responses to the *Nef* and *Gag* peptide pools respectively, with some IFN-ɣ responses above the 'positive' cut-off of >55 SFU/M PBMC (Table 6.2 and Figure 6.1). An ordinary one-way ANOVA comparing all 4 groups showed no statistical difference in the mean response at week 0 (p=0.7132).

Table 6.2: Summary of IFN-ɣ responses at week 0 (pre-vaccination) by peptide and group.

Week 0 Responders (N = number of participants)					
Peptide pool	**EP+IM (Active)**	**EP+IM (Placebo)**	**TC+IM (Active)**	**TC+IM (Placebo)**	**TOTAL**
Rev	2		1		**3/30 (10%)**
Tat	2				**2/30 (7%)**
Nef	8	4	8	**4**	**24/30 (80%)**
Gag	7	6	9	**4**	**26/30 (87%)**
CTL	5	3	4	**0**	**12/30 (40%)**

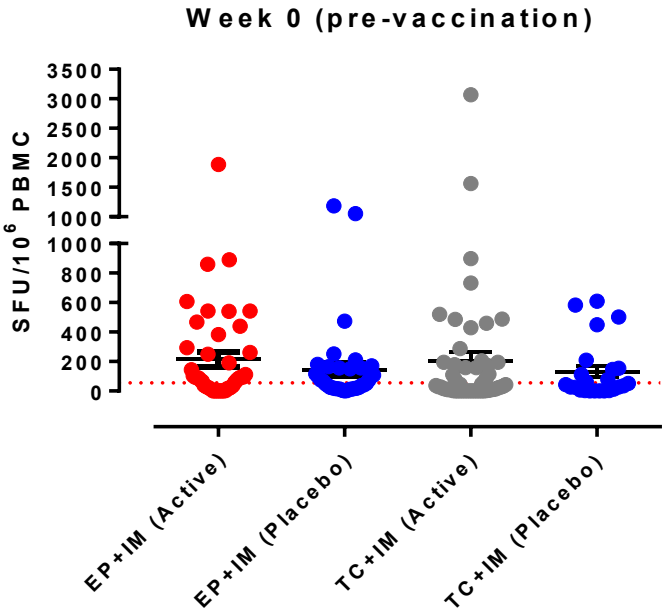


Figure 6.1: Scatter dot plot showing high baseline IFN-ɣ response at week 0 (pre vaccination) by group. All units are in SFU/M PBMC with background (from negative/mock wells) subtracted. The red dotted line represents the cut off of >55 SFU/M but participants also had to meet the second criteria to be a positive responders (>4 x baseline response if baseline response is more than 0).

6.2.2 Primary end point analysis (comparison of week 0 to week 14)

The primary end point in this trial was also at week 14, 2 weeks following the third and final vaccination. Before examining the fold increase in IFN-ɣ response, a few general comments can be made on the primary end point data for the 5 peptides pools *Rev, Tat, Nef, Gag, CTL.* The integrase peptide pool is discussed in a later section.

Analysis at the primary end point shows little variability in IFN-ɣ response either between or within each group in comparison to baseline

A comparison of the mean IFN-ɣ response at week 0 and the primary end point shows little variability in SFU/M PBMC with no significant difference comparing data from all groups at week 0 and week 14 (p=0.7335) (Figure 6.2).

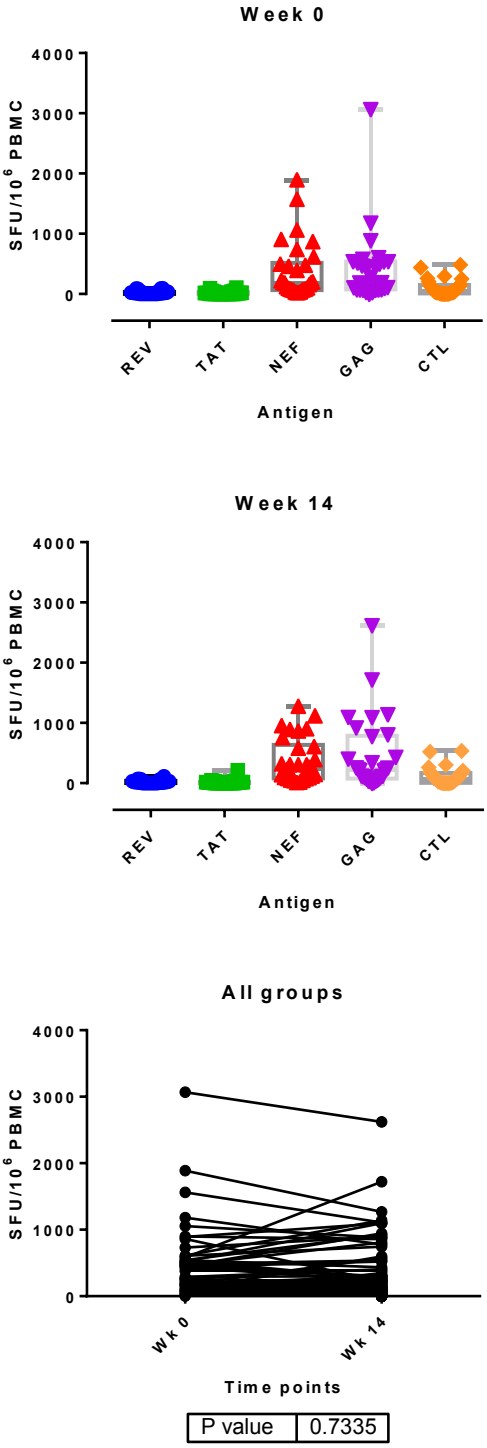


Figure 6.2: Box and whiskers graphs to show pooled IFN-ɣ response from all groups at week 0 and 14. The final figure is a before/after plot using a Wilcoxon matched-pairs signed t test to show no statistical difference between the IFN-ɣ magnitude at the pre vaccination time point and primary end point (all groups). Statistical significance was set as p=<0.05

At the primary end point, the mean magnitude of the IFN-ɣ response (shown in SFU/M PBMC) was greater across all peptide pools in the EP+IM (active) group, excluding *Gag*, with statistical significance comparing the difference in means for *Rev* (p=0.0453) and *Tat* (p=0.0466) (Table 6.3 and Figure 6.3).

However, a comparison of the IFN-ɣ response within each group at week 0 and week 14 showed no significant difference for any peptide at the primary end point in either the EP+IM (active) group (Figure 6.4) or TC+IM (active) group (Figure 6.5).

Table 6.3: INF-ɣ responses at week 14 shown by group and per peptide with mean, median and p values shown. Statistical significance is set at p=<0.05

	Group 1 EP+IM (Active)	**Group 2 TC+IM (Active)**	**Placebo (All)**	**Total (%)**
IFN-ɣ response at Week 14 (n/total)	4/9 (40%)	2/11 (18%)	3/10 (30%)	**9/30 All (30%)** **6/20 Active (30%)**
Rev (SFU/M)				**P Values (active only)**
Mean (SEM)	37.2 (9)	21.2 (11)	17.4 (6)	
Median	37	0	13	EP+IM vs TC+IM ***p=0.0453**
25-75 percentile	11-65	0-32	0-27	
Tat (SFU/M)				
Mean (SEM)	40.9 (22)	7.3 (4)	11.9 (4)	EP+IM vs TC+IM ***p=0.0466**
Median	20	3	10	
25-75 percentile	8-37	0-11	0-19	
Nef (SFU/M)				
Mean (SEM	421.7 (131)	386.1 (121)	305.0 (107)	EP+IM vs TC+IM p=0.5647
Median	298	203	205	
25-75 percentile	123-672	63-857	63-455	
Gag (SFU/M)				
Mean (SEM)	370.0 (130)	466.9 (234.5)	520.2 (181)	EP+IM vs TC+IM p=0.7103
Median	257	218	194	
25-75 percentile	80-679	37-405	92-892	
CTL (SFU/M)				
Mean (SEM)	158.2 (56)	133.8 (48)	42.1 (17)	EP+IM vs TC+IM p=0.7500
Median	103	68	19	
25-75 percentile	34-254	8-188	0-81	
Integrase (SFU/M)				
Mean (SEM)	110.4 (45)	165.5 (42)	181.7 (65)	EP+IM vs TC+IM p=0.3693
Median	52	203	85	
25-75 percentile	2-211	18-307	35-293	

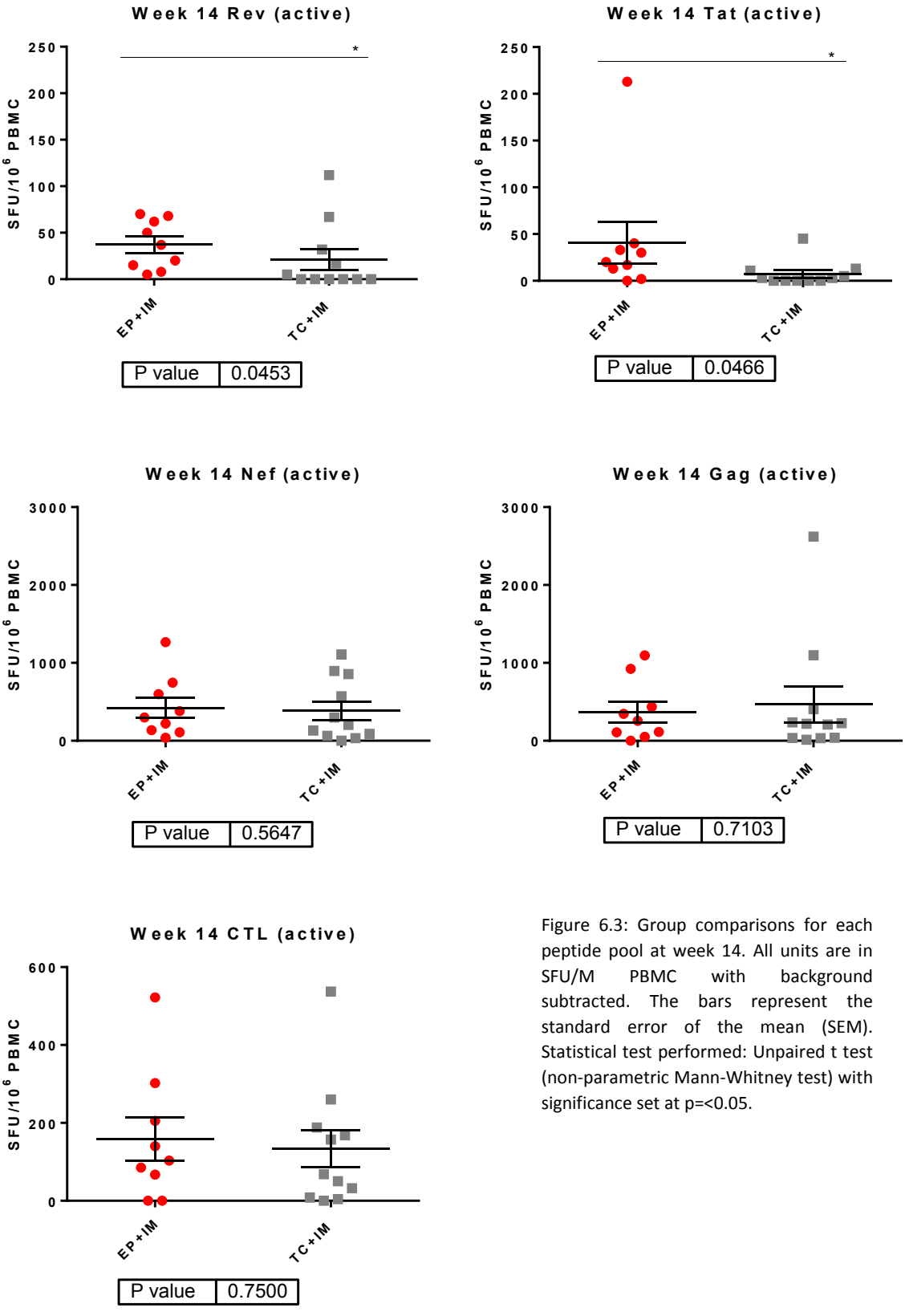


Figure 6.3: Group comparisons for each peptide pool at week 14. All units are in SFU/M PBMC with background subtracted. The bars represent the standard error of the mean (SEM). Statistical test performed: Unpaired t test (non-parametric Mann-Whitney test) with significance set at p=<0.05.

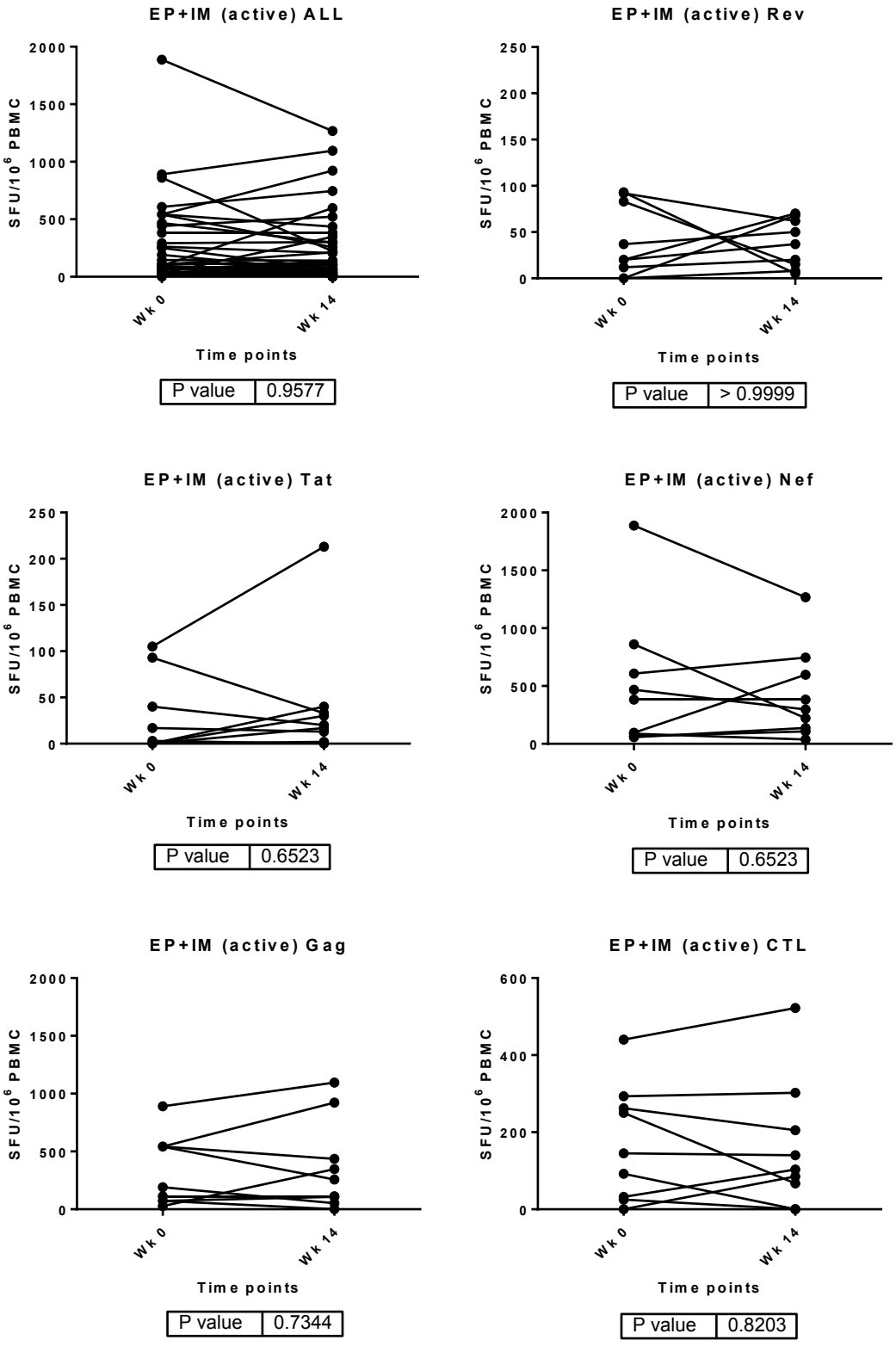


Figure 6.4: EP+IM group (active) Before/After graphs showing difference in magnitude of IFN-ɣ response from week 0 to week 14 initially with all peptide pools together (top left graph) and then individually. Statistical significance is set at p=<0.05. Each dot joined together by a line is an individual's IFN-ɣ response at week 0 and 14. Note differing scales on y axis indicating higher IFN-ɣ responses in *Nef* and *Gag* peptide pools.

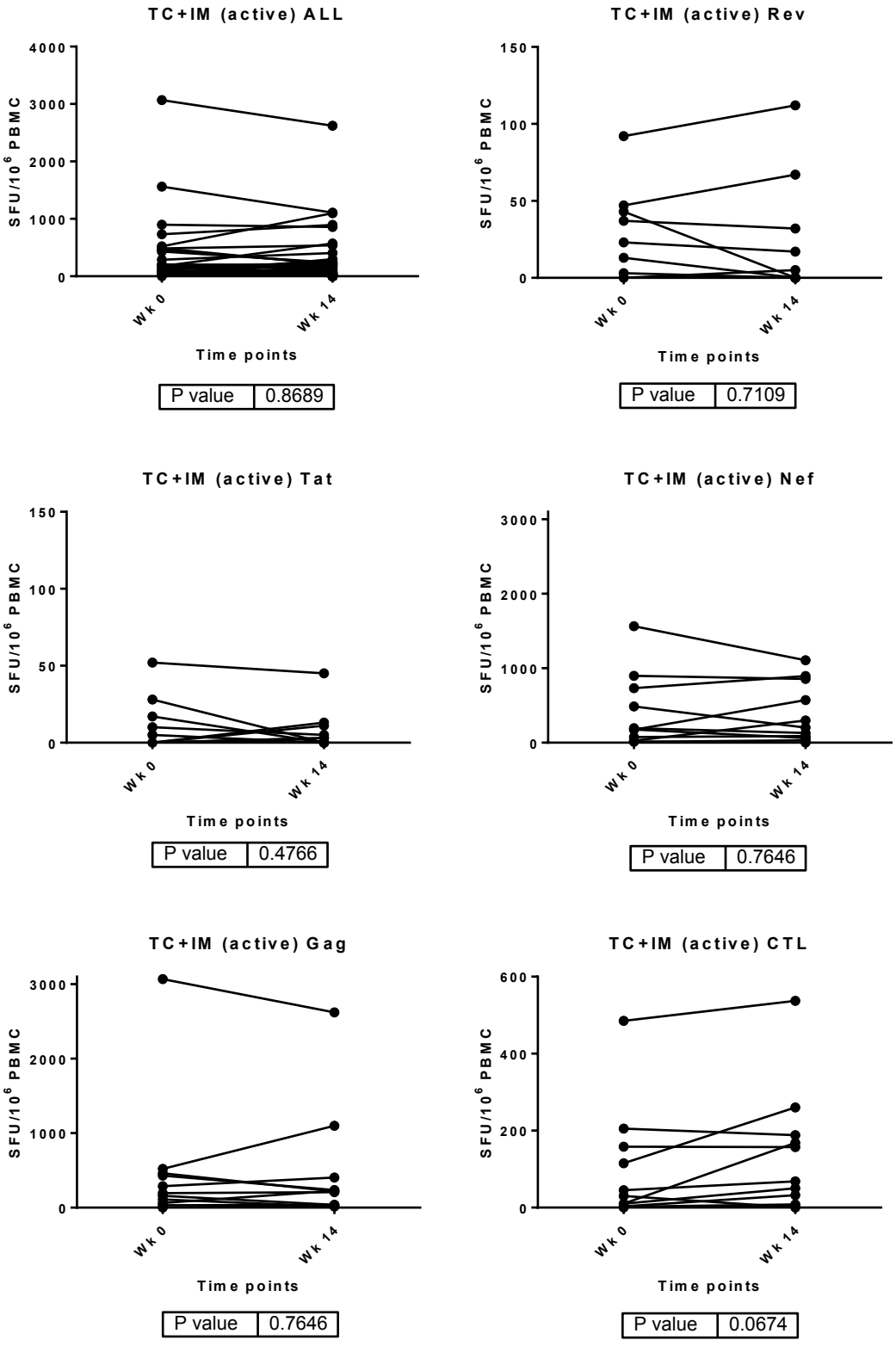


Figure 6.5: TC+IM group (active) Before/After graphs showing difference in magnitude of IFN-ɣ response from week 0 to week 14 initially with all peptide pools together (top left graph) and then individually. Statistical significance is set at p=<0.05. Each dot joined together by a line is an individual's IFN-ɣ response at week 0 and 14. Note differing scales on y axis indicating higher IFN-ɣ responses in *Nef* and *Gag* peptide pools.

Few participants within each active group showed a change in the IFN-ɣ response from baseline at the primary end point

As stated previously, the definition of a positive response for this trial was a doubling or 2-fold increase in frequency of IFN-ɣ from baseline (week 0). Should a baseline response be absent, then the same criteria as in CUTHIVAC 001 was applied which defines a positive response as >4 x mean background (if the background response is more than 0) and >55 background subtracted SFU/M.

In the EP+IM (active) group there were 4/9 participants (40%) who displayed either a 2 fold (or above) increase in IFN-ɣ at week 14 compared to week 0, or the presence of a response at week 14 which was absent at week 0 (Table 6.4 and Figure 6.6). Participants CT005 and CT017 both had a 2 fold increase in 1 peptide pool (*Nef* and *Tat* respectively), despite CT017 not receiving the third vaccine due to a new diagnosis of acute HCV. Participant CT011 had a positive response to both *Rev* and *CTL* peptide pools which was not present at baseline, and participant CT016 had a positive response to 2 peptide pools; *Nef* (6 fold increase from week 0) and *Gag* (response at week 14 which was absent at week 0).

In the TC+IM (active) group there were 2/11 (18%), participants who displayed increased IFN-ɣ responses from baseline meeting the pre-defined criteria above. Participants CT003 and CT032 both showed increases meeting the defined criteria above in response to 3 peptide pools; *Nef, Gag* and *CTL* (Table 6.4 and Figure 6.7).

With few responders in either of the active groups, it is difficult to make a comment about breadth of the IFN-ɣ response and any comment may be an over-interpretation of the results.

Table 6.4: Summary of responders at week 14 shown by group and to which peptide. A positive response was defined as a 2 fold increase at week 14 compared to week 0, or the presence of a response absent at baseline. To meet the latter criteria the IFN-ɣ response had to be >55 SFU/M if the baseline response was 0, or >4 x baseline response if above 0 and >55 SFU/M PBC.

Responders at week 14	Positive response to no. peptides at week 14	Comments
EP+IM (Active)		
CT005	1 *(Nef)*	2 fold increase
CT011	2 *(Rev, CTL)*	Negative to positive (both)
CT016	2 *(Nef, Gag)*	6 fold increase *(Nef)*; Neg to pos *(Gag)*
CT017	1 *(Tat)*	2 fold increase
TC+IM (Active)		
CT003	3 *(Nef, Gag, CTL)*	Neg to pos *(Nef)*; 3 fold increase *(Gag);* 2 fold increase *(CTL)*
CT032	3 *(Nef, Gag, CTL)*	3 fold increase *(Nef);* 2 fold increase *(Gag);* Neg to pos *(CTL)*
Placebo		
CT010 (EP+IM)	1 *(Nef)*	Negative to positive
CT012 (TC+IM)	1 *(Nef)*	2 fold increase
CT021 (TC+IM)	3 (*Rev, Nef, Gag,)*	Neg to pos *(Rev);* 2 fold increase *(Nef, Gag)*

Favourable HLA genotypes amongst participants displaying change from baseline at the primary endpoint

As discussed in the introduction to this chapter, HLA B*27, B*57, B*58:01, B*81:01 and B*51 have been identified as protective in HIV disease progression and important in viraemic control. In total, 8/30 (27%) participants were found to have favourable HLA genotypes. 3 of these participants showed changes in the IFN-ɣ response from baseline (Table 6.4). CT005, CT011 (both in the EP+IM active group) and CT032 (TC+IM active group) had favourable HLA I types HLA B*51, HLA B*51, HLA B*27 respectively (Figures 6.6 and 6.7). Therefore 50% of the responders across both groups were found to have a protective HLA allele known to be associated with favourable HIV-specific responses and protection against disease progression.

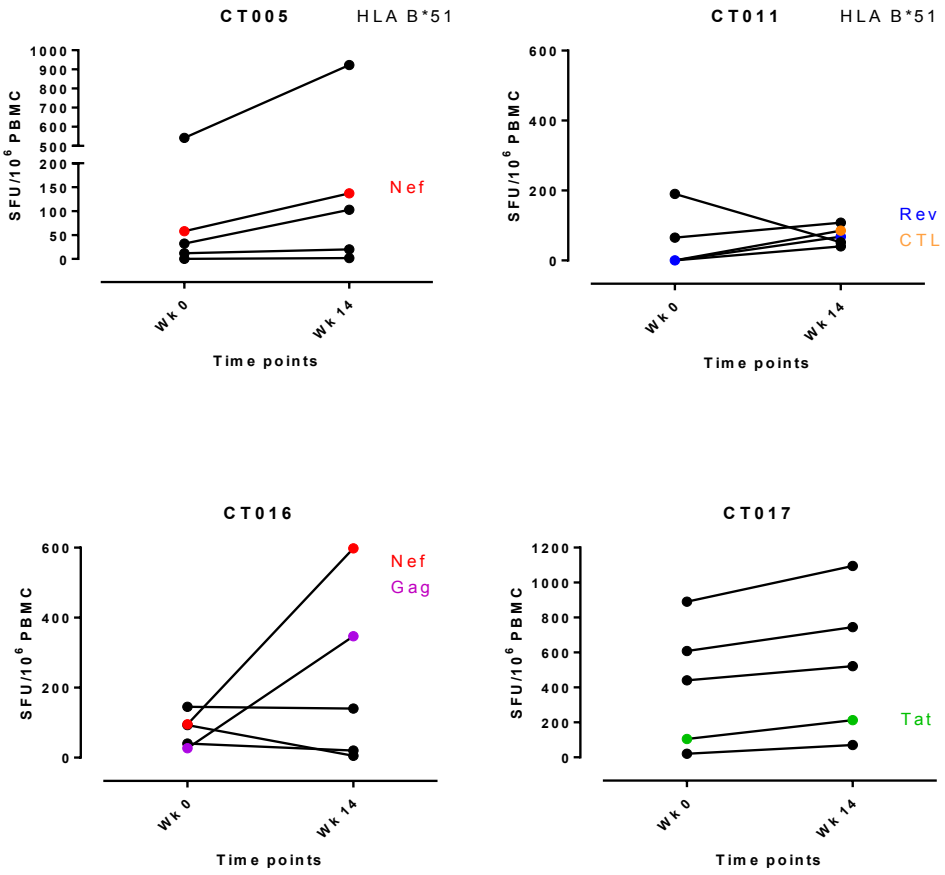


Figure 6.6: EP+IM (active) group positive responders by IFN-ɣ where each dot joined by a line is the participant's response to that particular peptide from week 0 to week 14. Peptide responses indicated and where appropriate the presence of a favourable HLA I type.

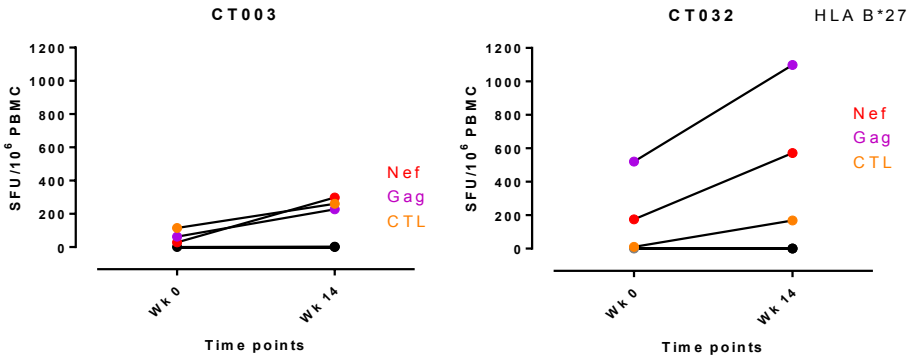


Figure 6.7: TC+IM (active) group positive responders by IFN-ɣ where each dot joined by a line is the participant's response to that particular peptide from week 0 to week 14. Peptide responses indicated and where appropriate the presence of a favourable HLA I type.

6.2.3 The placebo group: primary end point analysis

Through the process of randomisation, there were 6 participants in the EP+IM group and 4 participants in the TC+IM group receiving placebo (Chapter 5: Consort diagram).

Increased responses from baseline were also found amongst placebo recipients. In total there were 3 placebo recipients who displayed increased responses at week 14; CT010 in the EP+IM group (to *Nef*), CT012 in the TC+IM group (*Nef*), and CT021 also in TC+IM who responded to 3 peptides (*Rev, Nef, Gag*) (Table 6.4 and Figure 6.8). None of these participants had favourable HLA genotypes. The implications of these responses in the placebo group are considered in the discussion section.

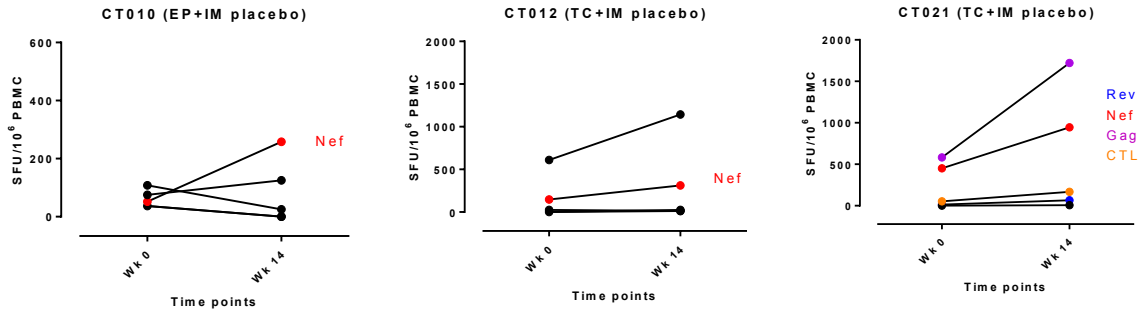


Figure 6.8: Positive responders by IFN-ɣ in placebo recipients where each dot joined by a line is the participant's response to that particular peptide from week 0 to week 14.

6.2.4 Responses to the integrase peptide pool at the primary end point

As stated in the methods section, the integrase peptide pool was not part of the vaccine construct, but was used in the ELISpot assay for this trial to determine if the mechanism of vaccination could trigger non-specific changes in immune function.

Data from week 0 to week 14 in participants receiving active drug showed no difference in the magnitude of the IFN-ɣ response to integrase, either for pooled group data or within the groups at these time points. In addition, at week 14 there were also no differences in magnitude comparing the groups (Figure 6.9).

In total, there were 5 participants showing a change in the IFN-ɣ response to the integrase peptide pool at week 14 (Figure 6.10). None were in the EP+IM active group, 3 in the TC+IM active group (CT013, CT029, CT032), and 2 in the placebo group (1 EP+IM placebo CT022, and 1 TC+IM placebo CT012). With so few responders to the integrase peptide pool, it is unlikely that vaccination has

resulted in non-specific immune changes in either group at the primary end point. The presence of responders to this peptide pool in placebo recipients raises questions of background variation or inflammation created by the vaccination process and is further examined in the discussion section.

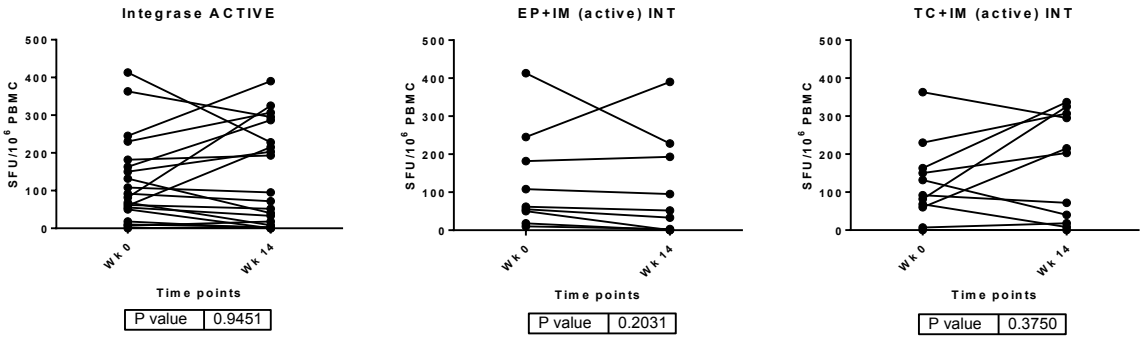


Figure 6.9: Before-after plots showing all data pooled (for both groups receiving active drug), and EP+IM (active) and TC+IM (active) groups at week 0 and week 14. Each dot joined by a line represents an individual participant's IFN-ɣ response at week 0 and 14. The Wilcoxon matched- pairs t test was used to determine statistical significance which was set at p=<0.05

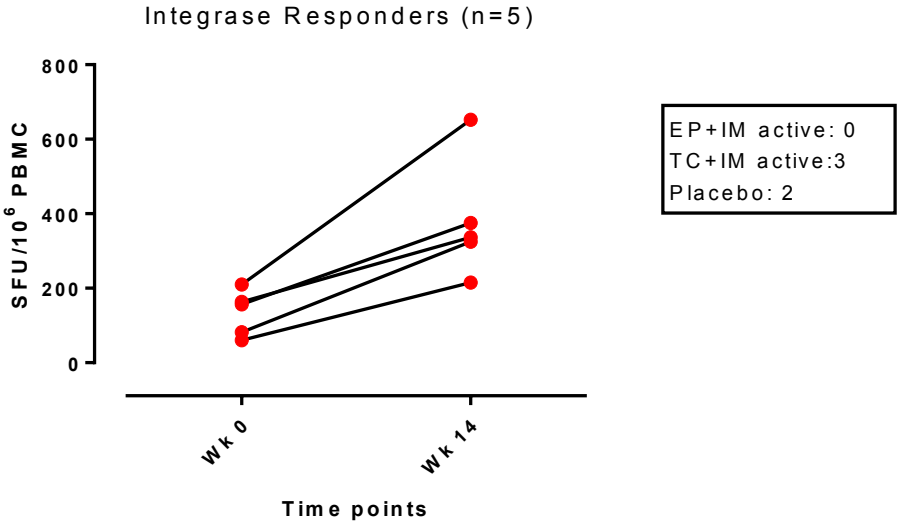


Figure 6.10: Before-after plot to show integrase responders at week 0 and week 14 and breakdown by group.

6.2.5 Interim time points (weeks 1 to 12)

PBMC samples were also analysed using the IFN-ɣ ELISpot assay at interim time points when participants were seen for their safety visits (see immunogenicity sampling schedule in methods section).

In the EP+IM (active) group there were a total of 6/9 (67%) responders at any time point including the primary end point. Similarly, in the TC+IM (active) group there were 7/11 (64%) responders at any time point (Table 6.5). There were also responders in both placebo groups, particularly in the TC+IM (placebo) group where all 4 placebo recipients showed a positive response to at least 1 peptide.

Table 6.5: Individual responders at each time point with response to peptide pool shown. Week 0 not shown as this has been shown in a previous table.

	EP+IM (Active)	**EP+IM (Placebo)**	**TC+IM (Active)**	**TC+IM (Placebo)**	**TOTAL**
Week 1	CT011 (*Nef*) CT016 (*Nef, Gag)* CT027 (*CTL*)		CT034 (*CTL*)		**4/30**
Week 4	CT005 (*Nef*) CT011 (*Nef*) CT016 (*Nef, Gag*)		CT018 (*Gag, CTL*) CT034 (*Tat*)	CT021 (*Rev, Gag)* CT012 (*Tat*)	**7/30**
Week 5	CT011 (*Rev*) CT016 (*Nef, Gag*)	CT025 (*Gag*)	CT018 (*CTL*)	CT012 (*Gag*) CT021 (*Gag*) CT028 (*Nef*)	**7/30**
Week 12	CT011 (*Gag*) CT016 (*Nef, Gag*)	CT022 (*Nef, Gag, CTL)* CT025 (*Gag*)	CT020 (*CTL*) CT026 (*Nef, Gag, CTL*) CT032 (*Nef, Gag, CTL*)	CT002 (*Nef, Gag)* CT028 (*Nef*)	**9/30**
Week 14	CT005 (*Nef*) CT011 (*Rev, CTL*) CT016 (*Nef, Gag*) CT017 (*Tat*)	CT010 (*Nef)*	CT003 (*Nef, Gag,CTL*) CT032 (*Nef, Gag, CTL*)	CT012 (*Nef*) CT021 (*Rev, Nef, Gag)*	**9/30**
Week 20	CT011 (*Rev, Nef, Gag)* CT017 (*Rev*) CT023 (*Gag*)	CT025 (*Gag*) CT030 (*CTL*)	CT003 (*Nef, Gag*) CT007 (*Gag*) CT018 (*Nef, CTL*) CT020 (*Gag)* CT032 (*Nef*)		**10/30**
Total no. **different** responders per group (%)	**6/9 (67%)**	**4/6 (67%)**	**7/11 (64%)**	**4/4 (100%)**	**21/30**

6.2.6 There were no differences in the duration of the IFN-ɣ responses between groups out to week 20

In this trial the final follow up point was at week 20, 8 weeks after the third vaccine. At week 20, and indeed across all time points shown, there were minimal differences between the EP+IM (active) and TC+IM (active) groups (Figure 6.11). The difference in mean IFN-ɣ magnitude between groups at week 20 did not reach statistical significance (p=0.5677).

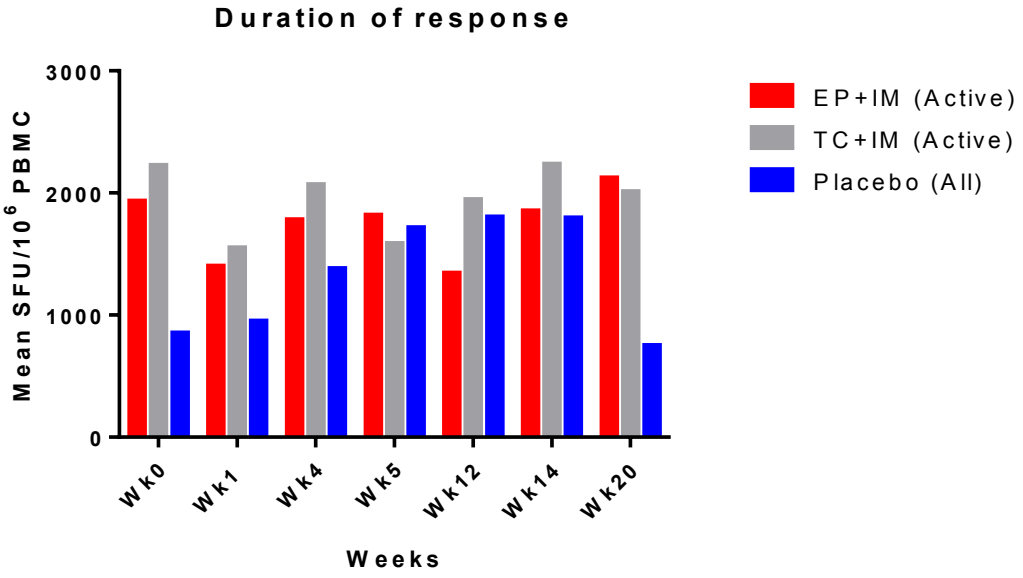


Figure 6.11: Bar chart showing mean SFU/M PBMC for all peptides pool together (excluding integrase). The x axis is time in weeks and the three groups are shown side by side.

A comparison of the magnitude of IFN-ɣ response within each group between week 0 and week 20 also showed no significant difference (Figure 6.12). Finally, a comparison of the integrase peptide pool at week 20 in each group compared to baseline was also unremarkable (Figure 6.13).

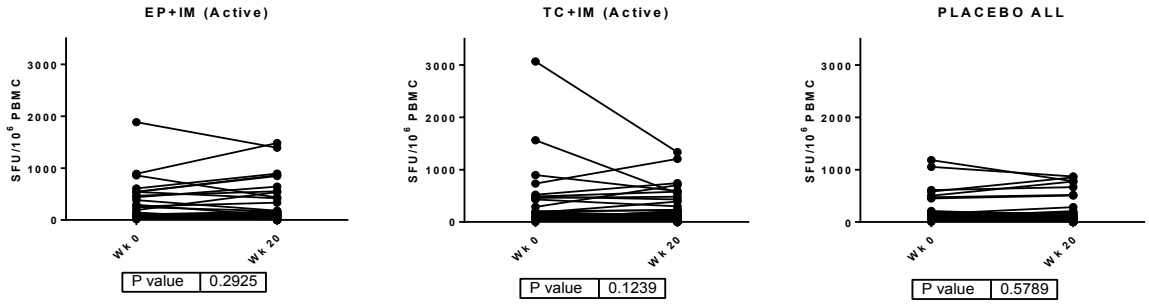


Figure 6.12: Group comparisons showing no statistical difference in IFN-ɣ response between weeks 0 and 20 in any group. For the placebo group both EP+IM and TC+IM placebo recipients are shown together. Statistical significance was set at p=<0.05.

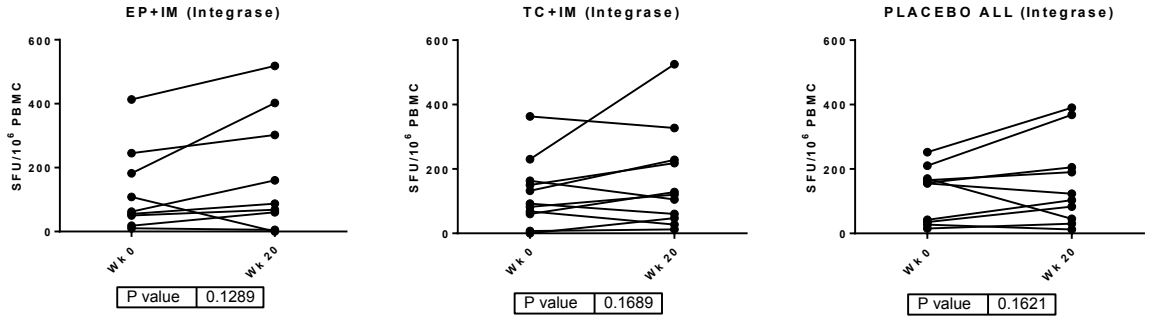


Figure 6.13: Group comparisons of the integrase peptide pool showing no statistical difference in IFN-ɣ response between weeks 0 and 20 in any group. For the placebo group both EP+IM and TC+IM placebo recipients are shown together. Statistical significance was set at p=<0.05.

6.2.7 Comparison of IFN-ɣ response at baseline in this trial (CUTHIVTHER Week 0) with primary end point IFN-ɣ response in vaccinated uninfected participants (CUTHIVAC Week 14)

To provide a complete analysis on immunogenicity, the IFN-ɣ responses in this trial were compared to the previous trial (CUTHIVAC 001) in HIV negative participants. Whilst putting this data together it was noticed the magnitude of the IFN-ɣ response at week 0 in this trial was comparable to the primary end point magnitude CUTHIVAC 001, therefore I sought to determine if comparisons could be drawn with respect to immune function between the two trials, despite the goal of vaccination in these two cohorts being different.

A comparison of week 0 IFN-ɣ pre-vaccination responses in this trial to the primary end point response in the CUTHIVAC trial showed a significant difference in magnitude of the IFN-ɣ response at baseline in HIV infected volunteers (all peptides and groups pooled together), suggesting the presence of pre-existing responses to the peptide pools (Figure 6.14 and Table 6.6). When this is broken down by peptide pool for the same time points, the greatest difference in magnitude is for the *Nef* and *Gag* peptide pools (Figure 6.15). The potential relevance of this is addressed in the discussion section.

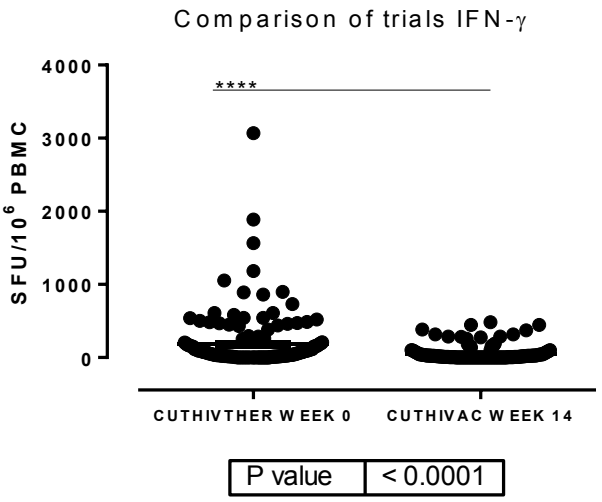


Figure 6.14: Comparison of the IFN-ɣ response at baseline (week 0) in the CUTHIVTHER trial of HIV infected volunteers and the primary end point (week 14) response in the CUTHIVAC trial. The data shown here is all participants and peptides pooled together. Statistical significance was set at p=<0.05.

Table 6.6 Comparison of the IFN-ɣ response at baseline (week 0) in the CUTHIVTHER trial of HIV infected volunteers and the primary end point (week 14) response in the CUTHIVAC trial. The data shows the pooled mean, median and interquartile range (SFU/M PBMC).

SFU/Million PBMC (all data pooled)	CUTHIVTHER Week 0 (HIV infected participants)	CUTHIVAC Week 14 (HIV uninfected participants)
Mean	181.9	53.8
Median	52.0	14.0
25%-75% percentile	12.3-173.8	1.5-42.3

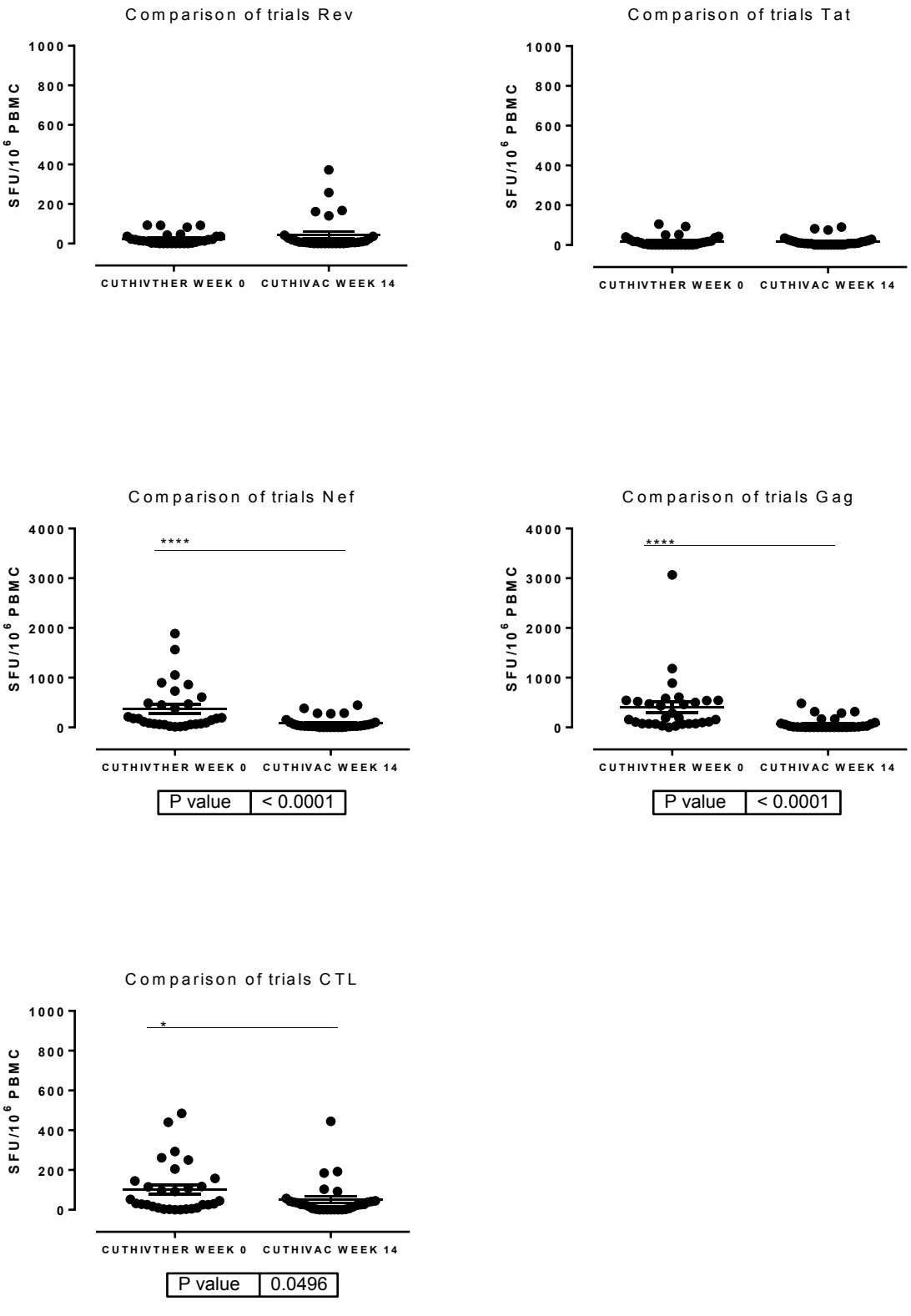


Figure 6.15: Comparison of the magnitude of IFN-ɣ response at baseline (week 0) in the CUTHIVTHER trial of HIV infected volunteers and the primary end point (week 14) response in the CUTHIVAC trial. The data shown here is for all participants

to individual peptide pools. Statistical significance was set at p=<0.05. Note the differing scales for *Nef* and *Gag* indicating a greater magnitude of the IFN-ɣ response in these peptide pools.

6.2.8 Change in CD4+ count at week 14 from baseline and comparison to FIT-06 clinical trial using GTU®MultiHIV B clade DNA vaccine in HIV infected participants not on ART

As stated in the introduction to this chapter, the FIT-06 clinical trial in South Africa enrolled Clade C HIV infected participants with relatively high CD4+ counts at enrolment (enrolment criteria >350 cells/mm3 at screening) [135]. In the FIT-06 study participants were not on ART, a major difference to CUTHIVTHER where all participants had undetectable VLs on treatment and therefore comparisons on HIV-RNA cannot be drawn between the two clinical trials.

However, FIT-06 reported significant changes in CD4+ cells in the group receiving IM DNA vaccination compared to placebo throughout the study period, with a total increase of 73 cells/mm^3 for pooled data (p-0.012). Some comparisons with CUTHIVTHER can be drawn taking into account CD4+ counts at week 0 and week 14.

In CUTHIVTHER, the mean CD4+ count at week 0 was higher at 753 compared to 346 in FIT-06. FIT-06 also reported an average CD4+ increase of 73 cells/mm^3 throughout the trial, whereas in CUHIVTHER there was no change in total CD4+ count across groups at the primary end point (week 0 mean all groups 753 cells/mm^3, week 14 mean all groups 745 cells/mm^3) (Table 6.7). Furthermore there were no significant differences found for pooled CD4+ data at the primary end point, nor when broken down for each group and compared to baseline (Figure 6.16). Reasons for this discrepancy across these two clinical trials using the same vaccine as discussed later in this chapter.

Table 6.7: Mean CD4+ counts with standard deviations shown by group at week 0 and week 14

Group	Mean CD4+ count Week 0	Standard Deviation Week 0	Mean CD4+ count Week 14	Standard Deviation Week 14
EP+IM Active	807	220	844	265
EP+IM Placebo	818	140	767	289
TC+IM Active	709	224	710	200
TC+IM Placebo	677	108	659	137
Total (all grps)	753		745	

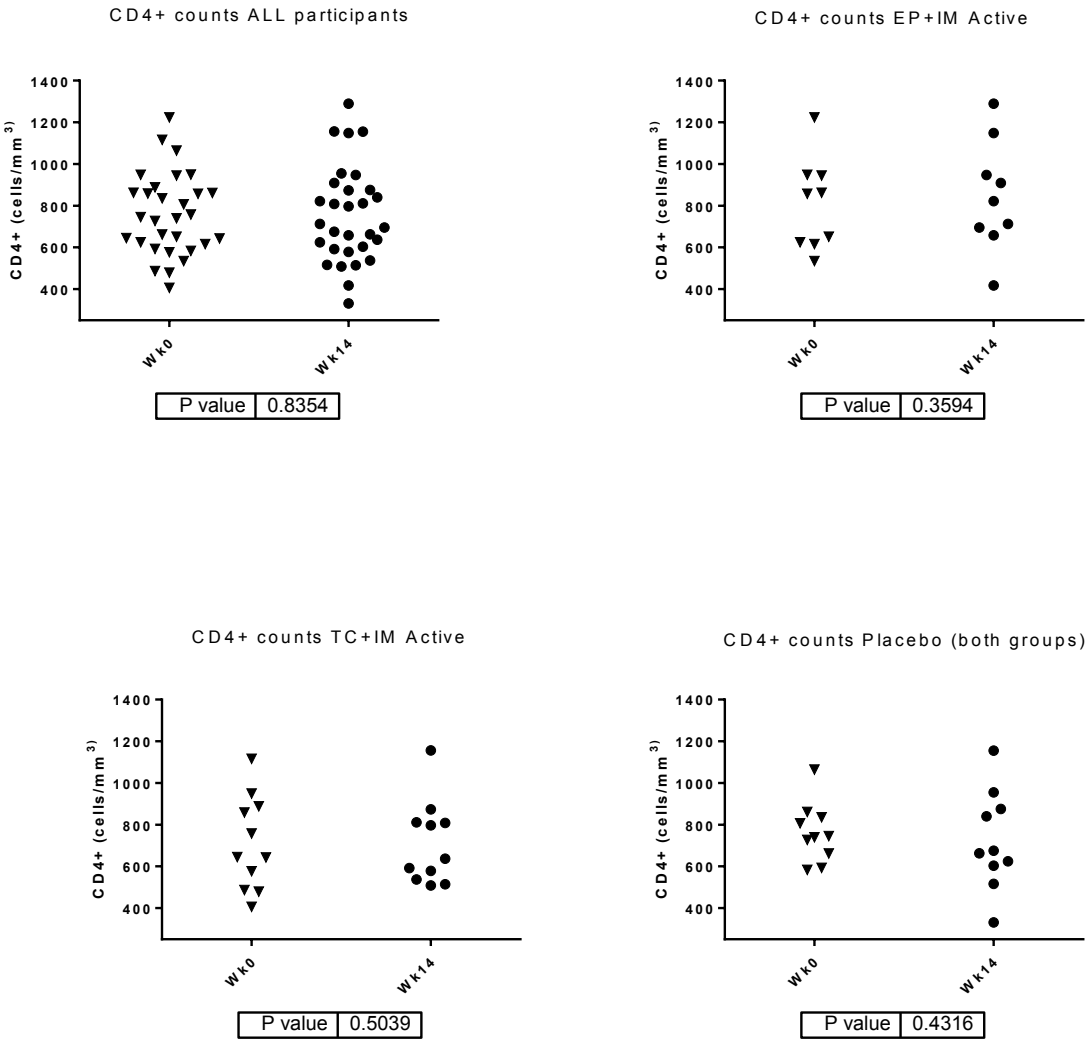


Figure 6.16. Scatter dot plots to show total CD4+ cell counts at week 0 and week 14 with p values and groups shown. Statistical significance was set at p=<0.05.

Summary of Immunogenicity results: T cell IFN-ɣ ELISpot responses

This section demonstrates although there were some changes in the IFN-ɣ response from baseline in a few participants, DNA vaccination was not able to induce HIV-specific immunity in this HIV positive cohort and the T cell response did not seem to be influenced by the route of vaccine administration

- There was a high degree of pre-vaccination IFN-ɣ responses in this HIV positive cohort, particularly to the *Nef* and *Gag* peptide pools
- Analysis at the primary end point showed little variability in IFN-ɣ response either between or within each group in comparison to baseline, although there were a small number of participants in each active group demonstrating a change in the IFN-ɣ response from baseline
- Of the participants showing a change in the IFN-ɣ from baseline, 50% had favourable HLA genotypes
- IFN-ɣ responses at week 14 were also noted in participants from the placebo groups
- It is unlikely vaccination via either the transcutaneous or electroporation in combination with IM resulted in non-specific immune changes as displayed in results from the integrase peptide pool
- There were no differences in the duration of the IFN-ɣ responses between groups out to week 20
- A comparison of week 0 IFN-ɣ pre-vaccination responses in this trial to the primary end point response in the CUTHIVAC trial showed a significant difference in magnitude of the IFN-ɣ response at baseline in HIV infected volunteers
- Overall there was little change in CD4+ counts between week 0 and week 14 in any group

6.3 SUPPORTING WORK: DATA ANALYSIS FROM ICS

As in the previous results chapter, to examine the quality of the cellular immune response, an analysis of the results of vaccine-induced T cells by intracellular cytokine staining (ICS) was conducted.

The ICS data is presented as a percentage total of CD4+ or CD8+ cells with all background responses from negative controls subtracted. For the CD4+ results data for cytokines IFN-ɣ, IL-2, TNF-α and CD154 is presented, and for CD8+ cytokines IFN-ɣ, IL-2, TNF-α, CD154, granzyme B and perforin.

6.3.1 Primary end point comparison of ICS data between groups showed few changes from baseline in either group

At week 14, the CD4+ ICS data for IFN-ɣ showed a significant difference in the *gag*-specific IFN-ɣ response in the TC+IM (active) group compared to the EP+IM (active) group (p=0.0014) (Figure 6.17). There were no differences for any peptide for IL-2 or TNF-α between the groups (Figures 6.18 and 6.19). The CD4+ data for CD154 similarly showed a statistically significant difference in the TC+IM (active) group compared to the EP+IM (active) group for the *Rev* and *Gag* peptide pools (p=0.0487, p=0.0264 respectively) (Figure 6.20).

For the CD8+ data the TC+IM (active) group again showed statistically significant differences for *Nef*-specific and *CTL*-specific peptide responses to IFN-ɣ (p=0.0262, p=0.0248 respectively), *Tat*-specific and *Nef*-specific responses to TNF-α (p=0.0008, p=0.0052), and *CTL*-specific response to CD154 (p=0.0117) (Figures 6.21-6.23). There were no differences between the groups for any peptide specific response to IL-2, granzyme B or perforin (Figures 6.24-6.26).

6.3.2 Primary end point comparison of ICS data within each group between week 0 and week 14 shows few changes from baseline to any peptide, with most changes in the TC+IM (active) CD8+ data

CD4+

Comparisons of pre-vaccination (week 0) and primary end point data (week 14) within each group were minimal with the only statistically significance change in response in the TC+IM (active) group in the *CTL* peptide pool to IFN-ɣ (p=0.0088) (Appendix 7.2). There were no other significant responses seen in any group to IL-2, TNF-α or CD154.

CD8+

Comparisons of pre-vaccination (week 0) and primary end point data (week 14) within each group showed significant responses particularly in the TC+IM (active) group. In this group, for IFN-ɣ there was a statistically significant response at week 14 in the *CTL* peptide pool (p=0.0029) (Appendix 7.5), for IL-2 in the *Gag* and *CTL* peptide pools (p=0.0391 and p=0.0078 respectively) (Appendix 7.7), and for CD154 also in the *Gag* and *CTL* peptide pools (p=0.0322 and p=0.0049 respectively) (Appendix 7.8). There was also a difference noted in the placebo group for CD154 in the *Tat* and *Gag* peptide pools (p=0.0059) (Appendix 7.9). There were no other significant responses found at week 14 across any peptide pool to any cytokine.

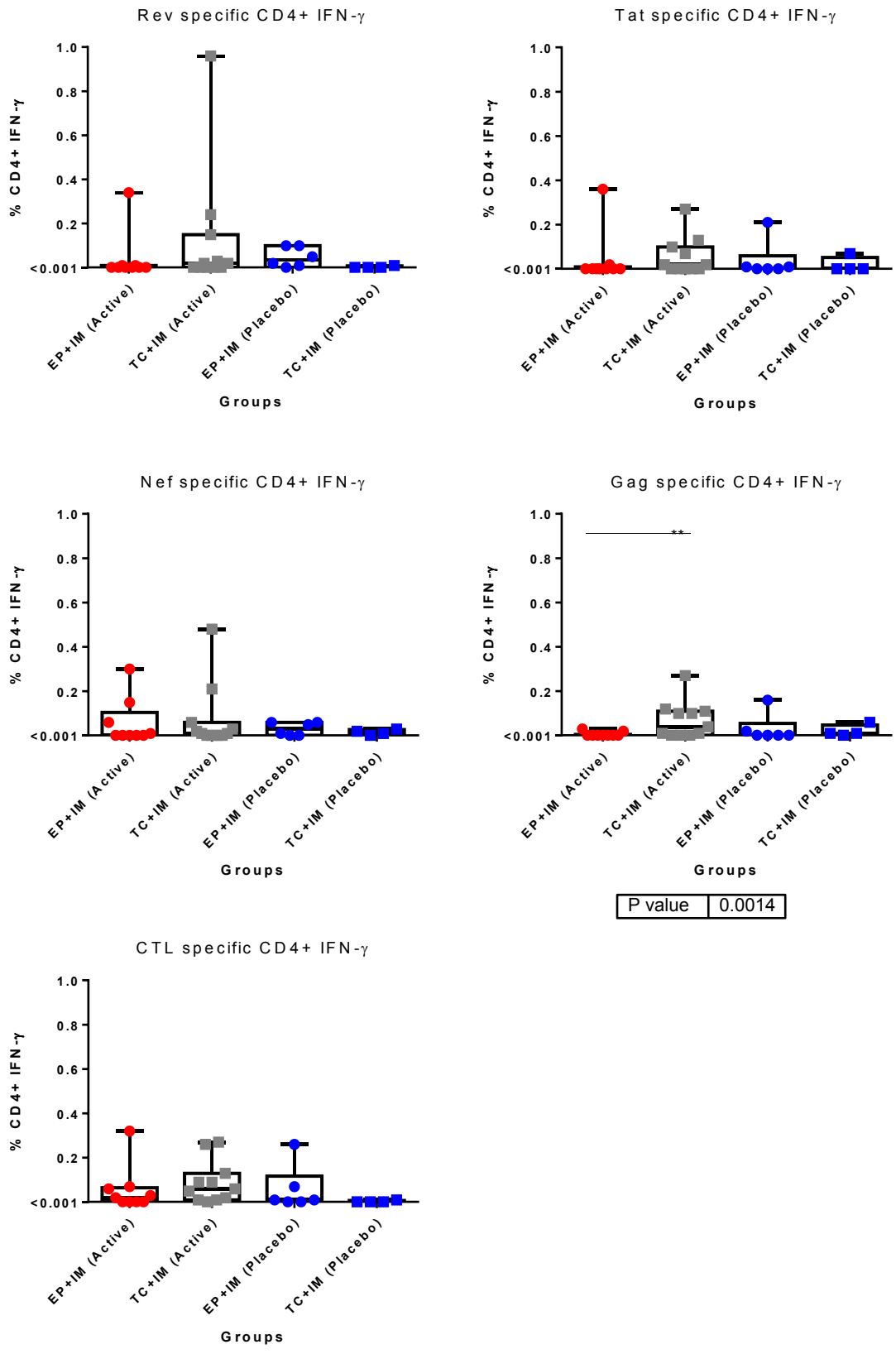


Figure 6.17: CD4+ IFN-ɣ response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

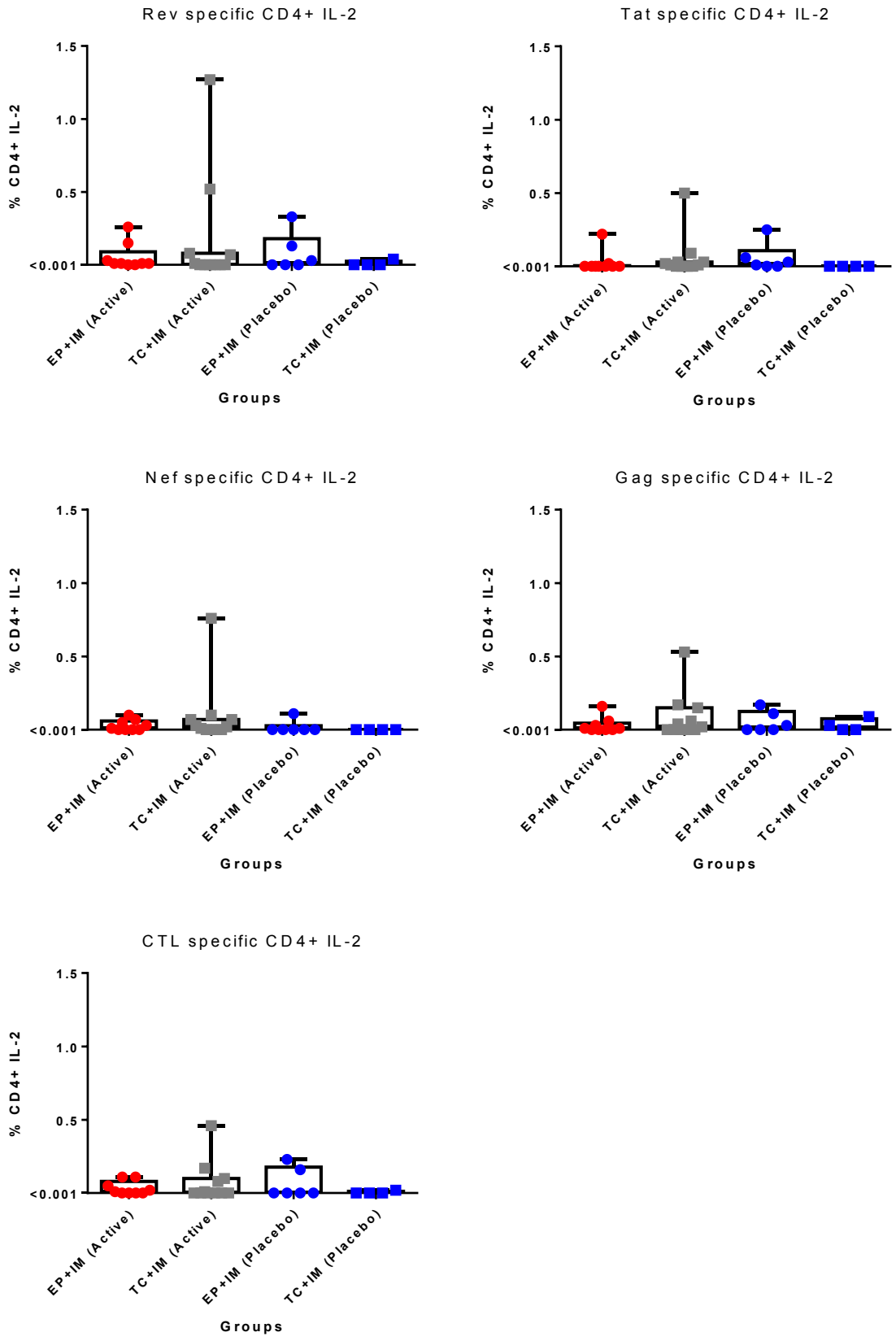


Figure 6.18: CD4+ IL-2 response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

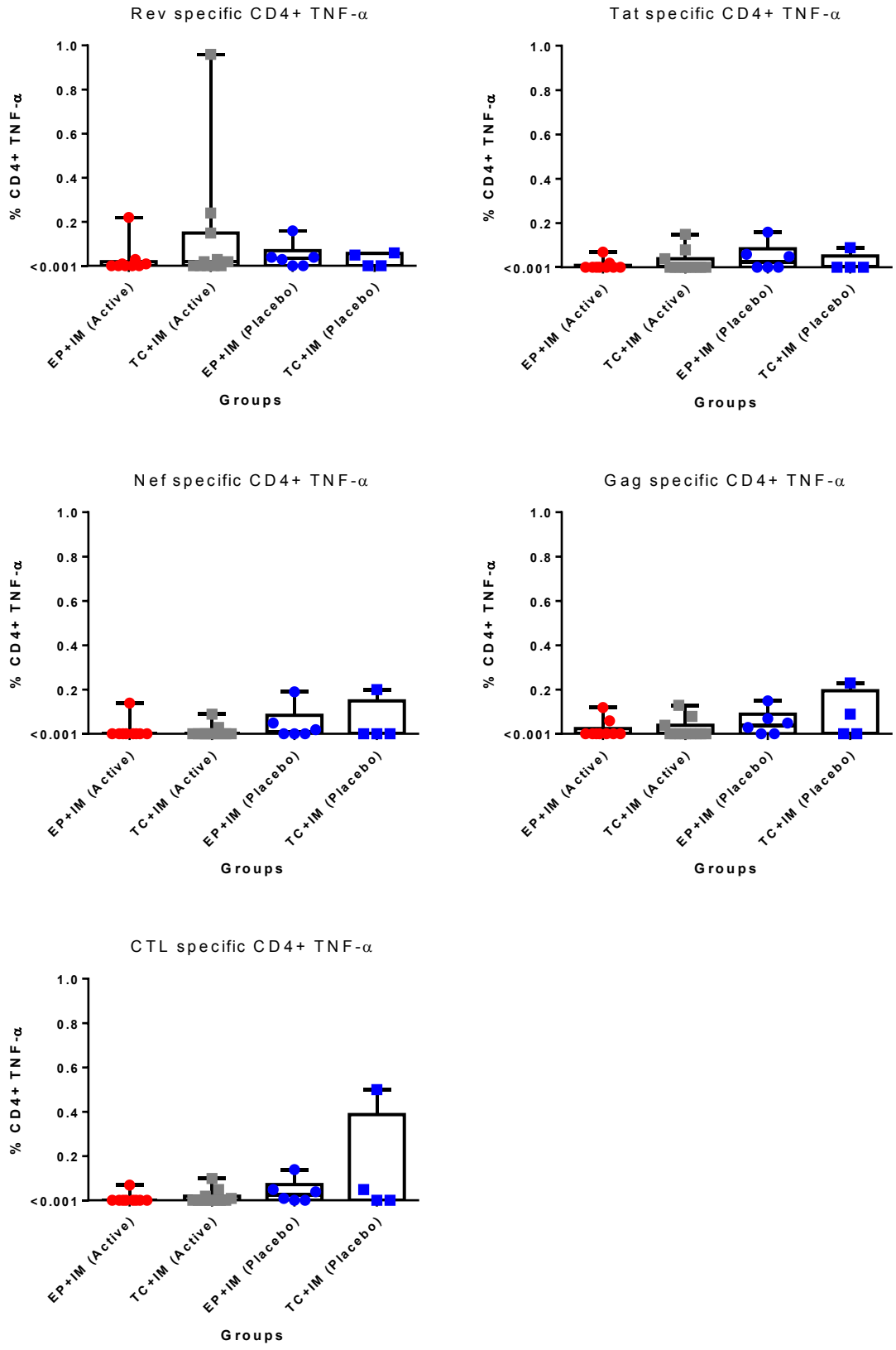


Figure 6.19: CD4+ TNF-α response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

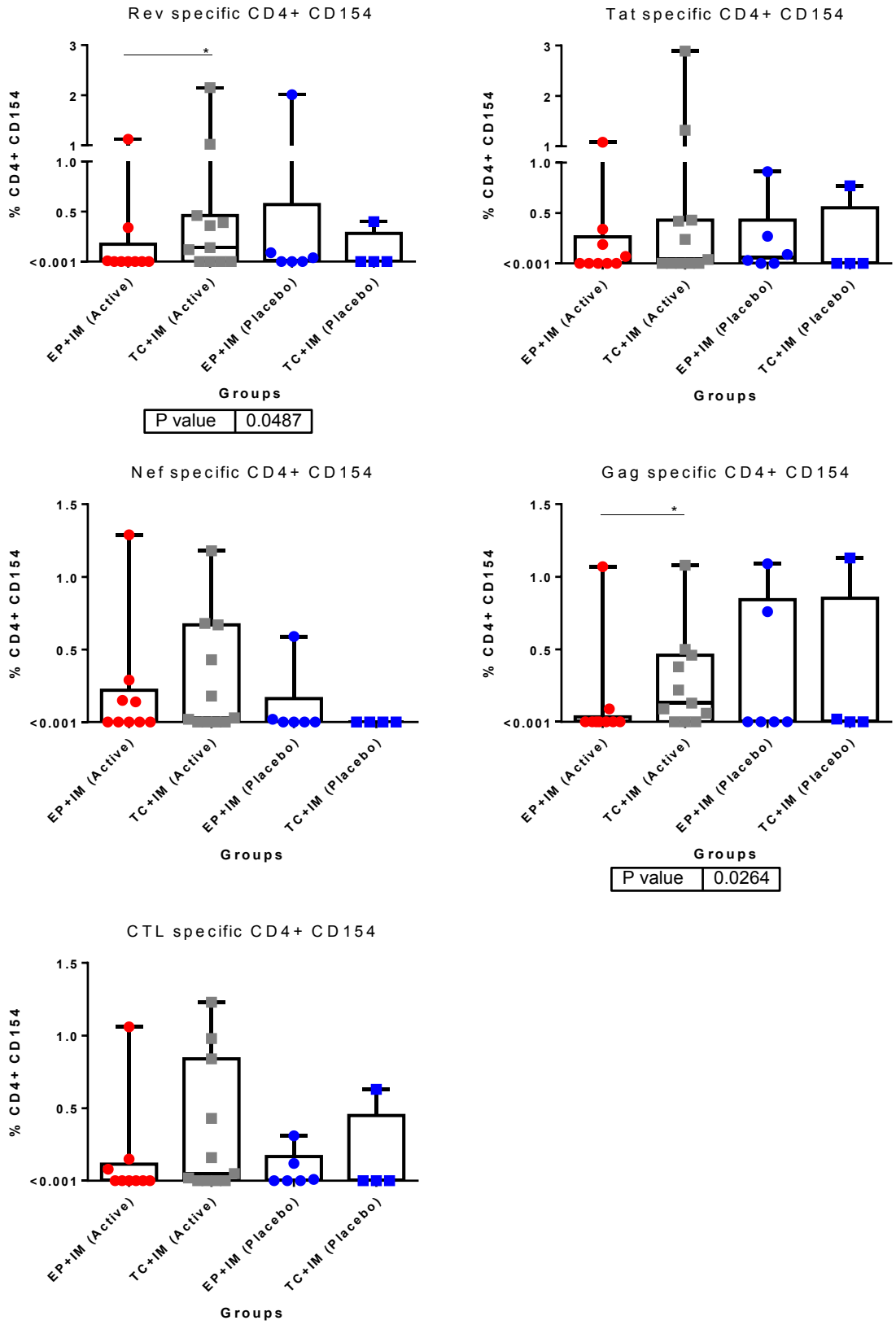


Figure 6.20: CD4+ CD154 response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

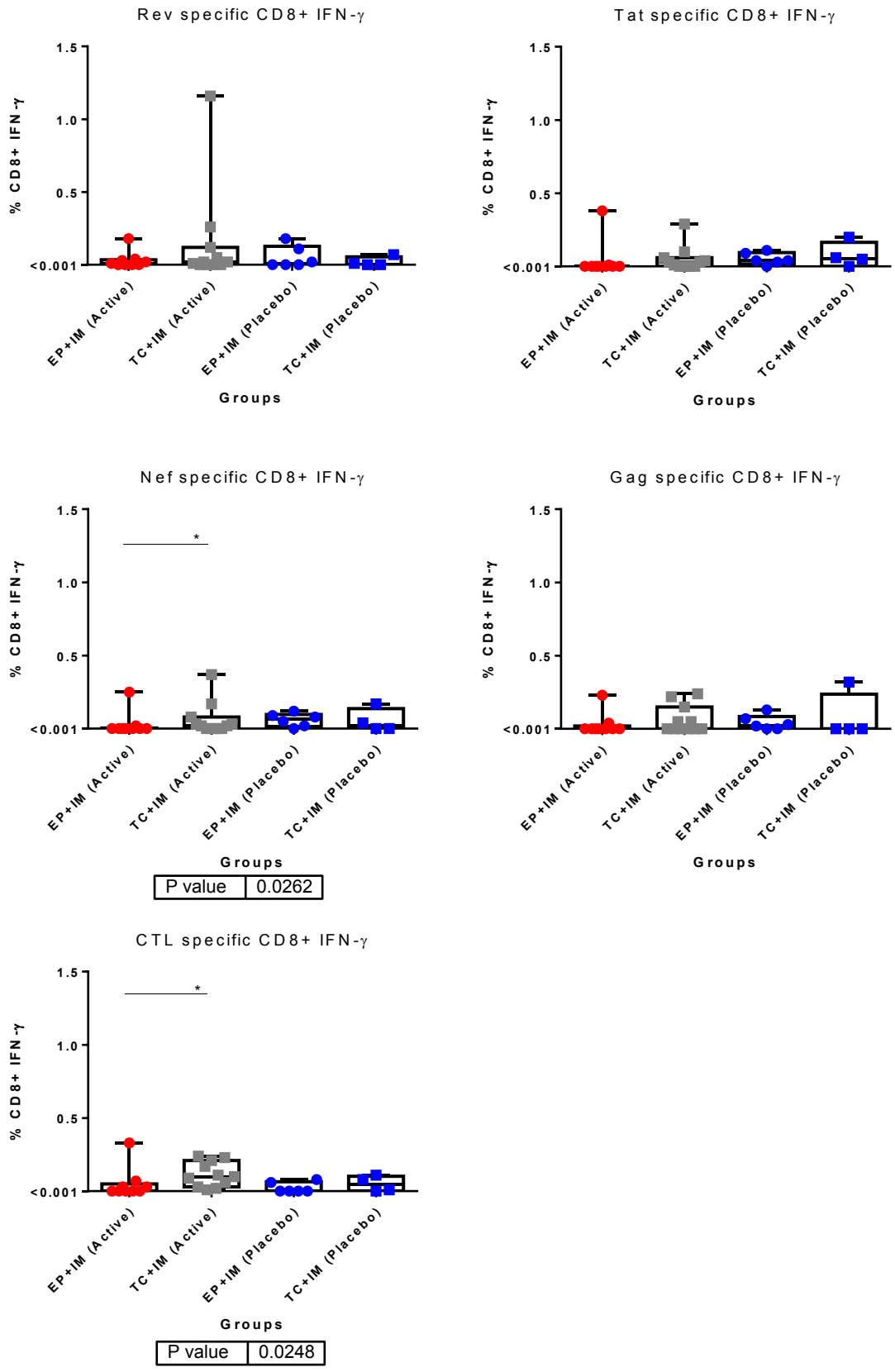


Figure 6.21: CD8+ IFN-ɣ response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

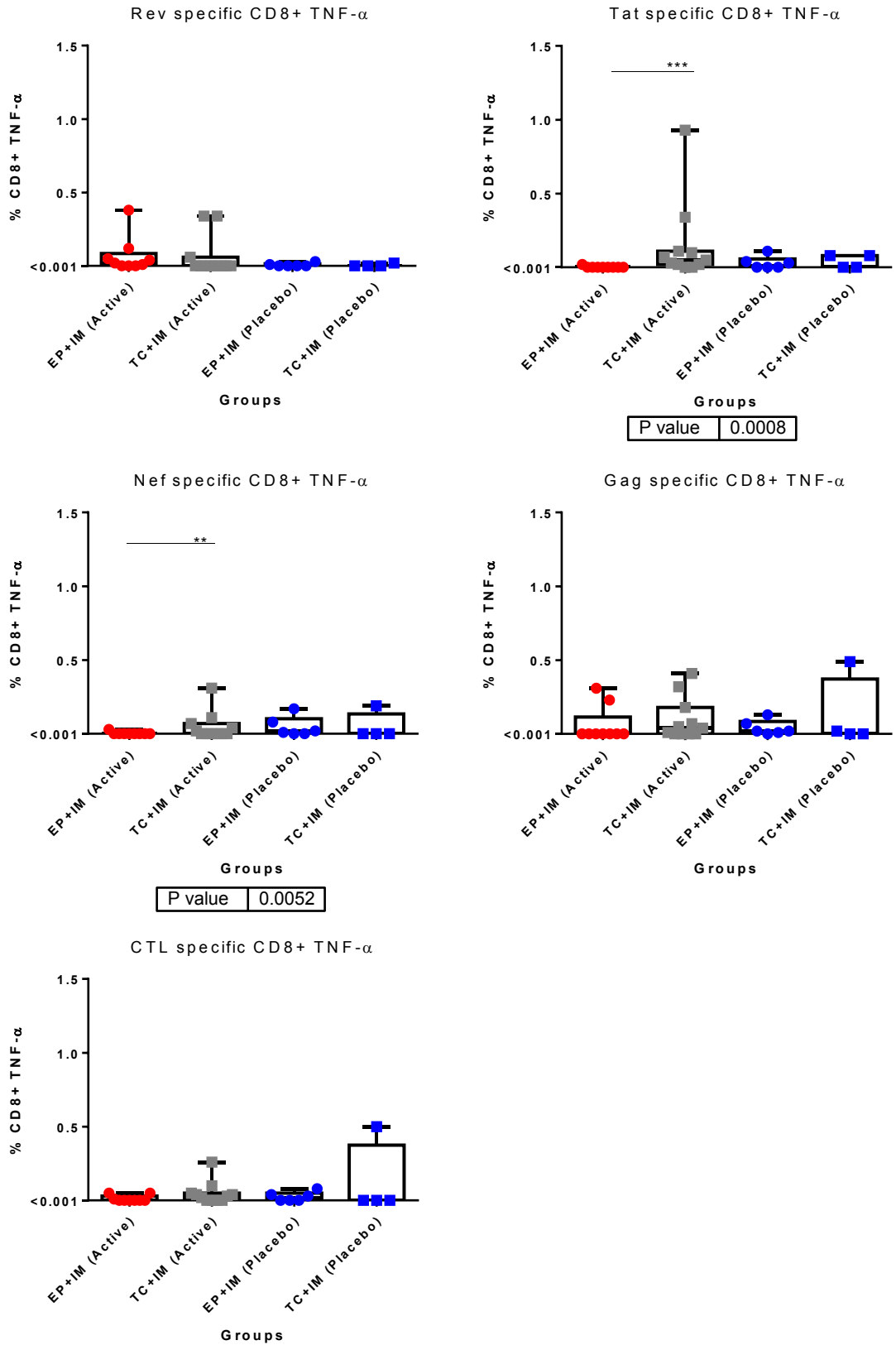


Figure 6.22: CD8+ TNF-α response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

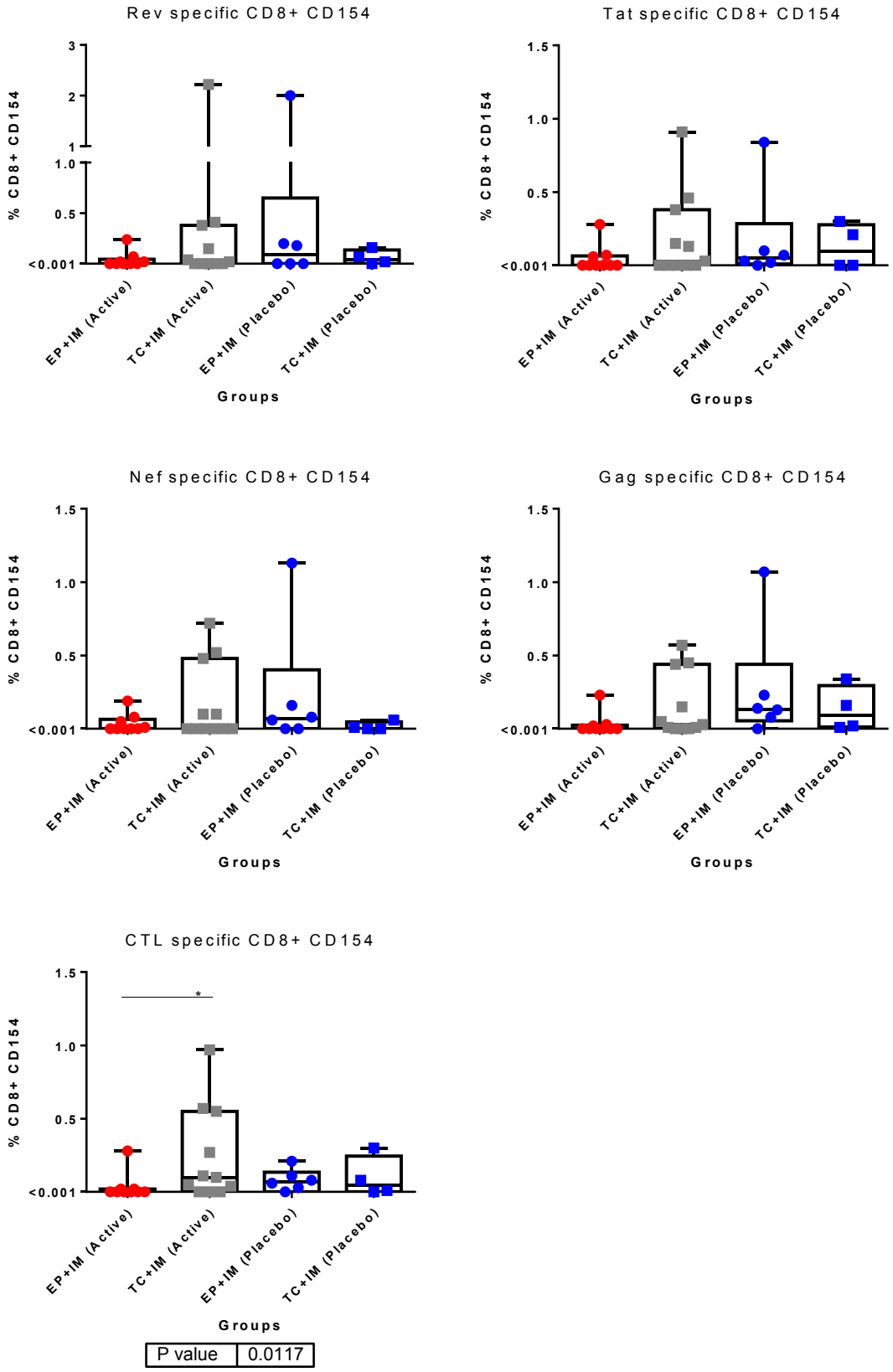


Figure 6.23: CD8+ CD154 response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

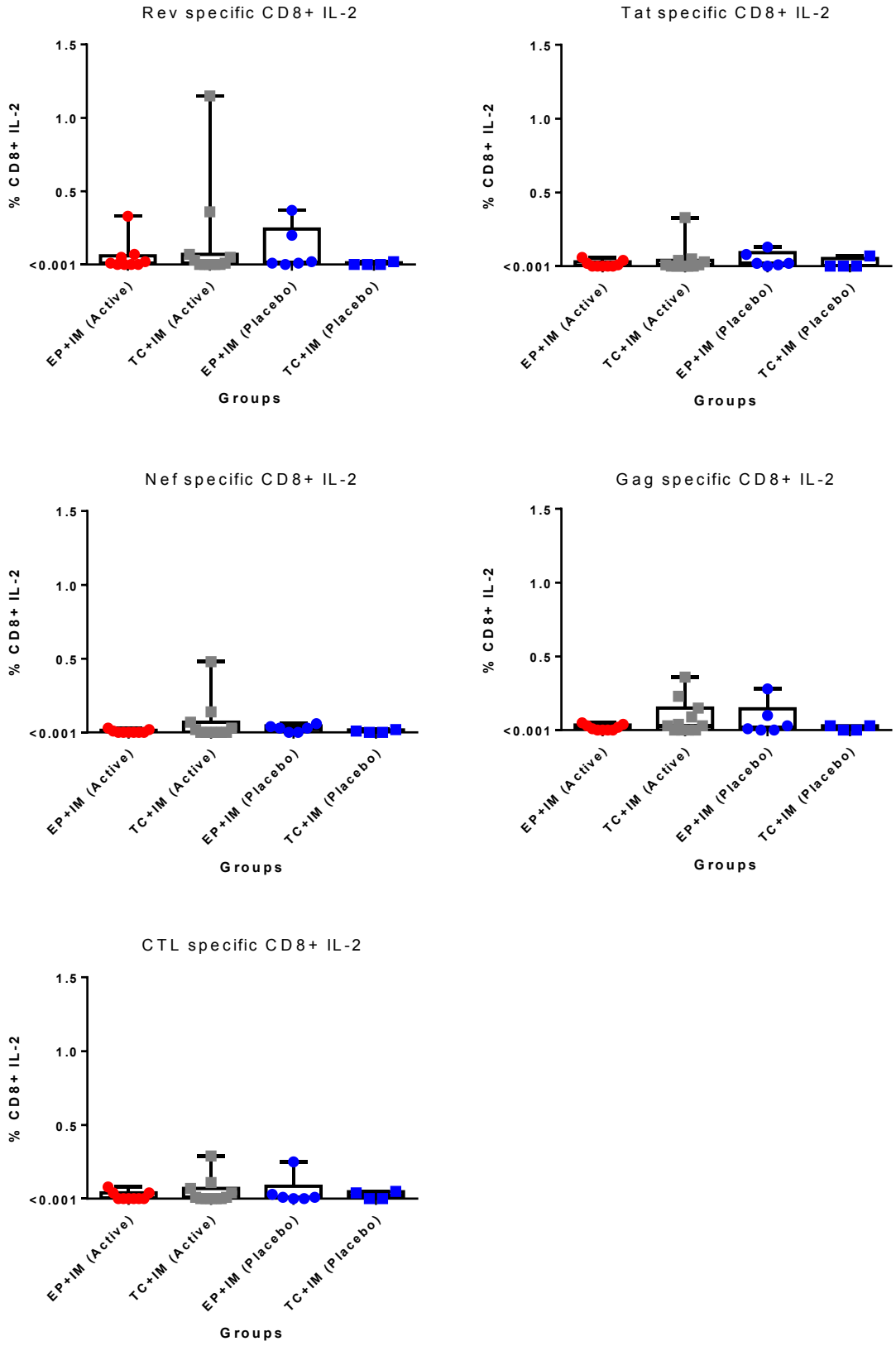


Figure 6.24: CD8+ IL-2 response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

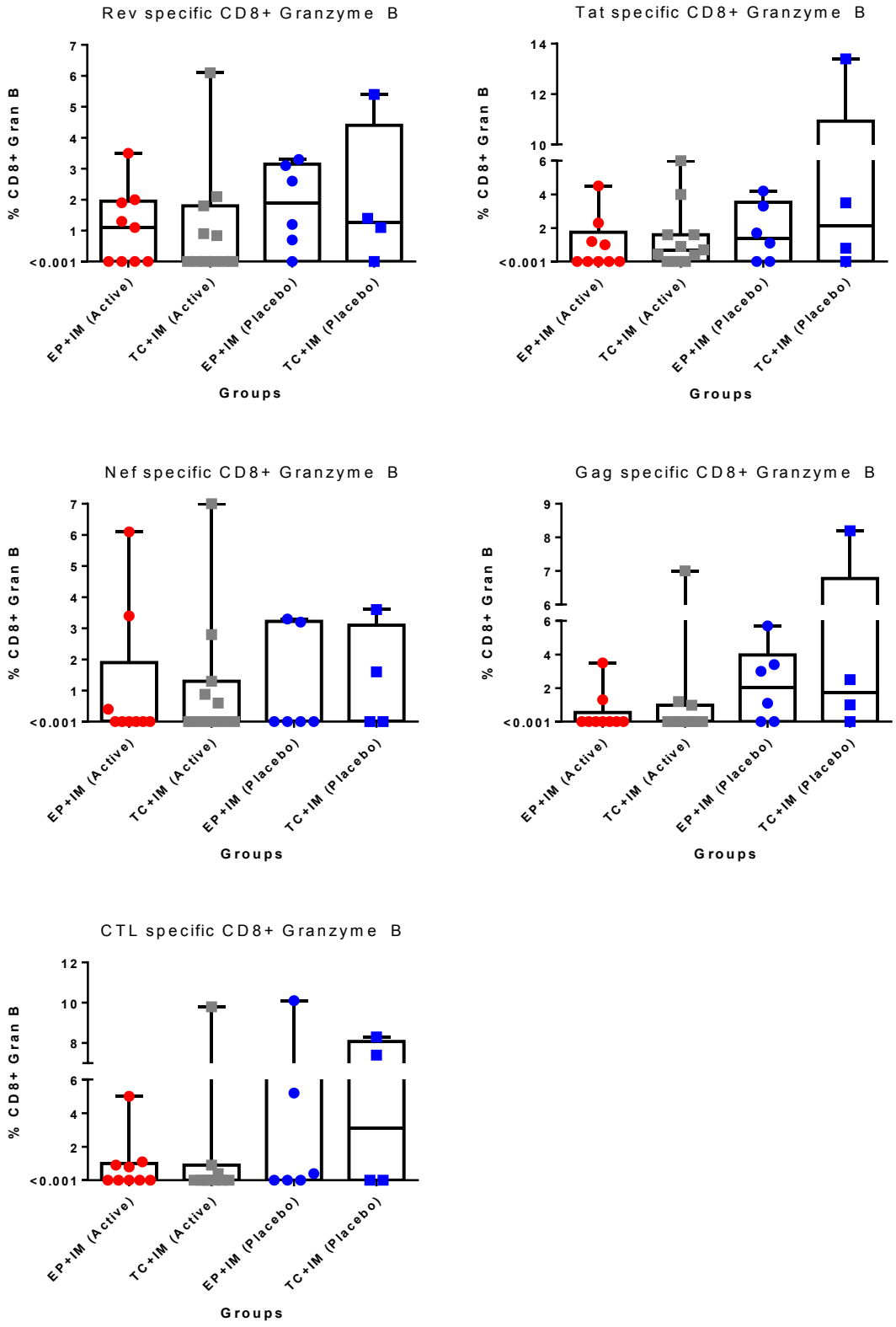


Figure 6.25: CD8+ granzyme B response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

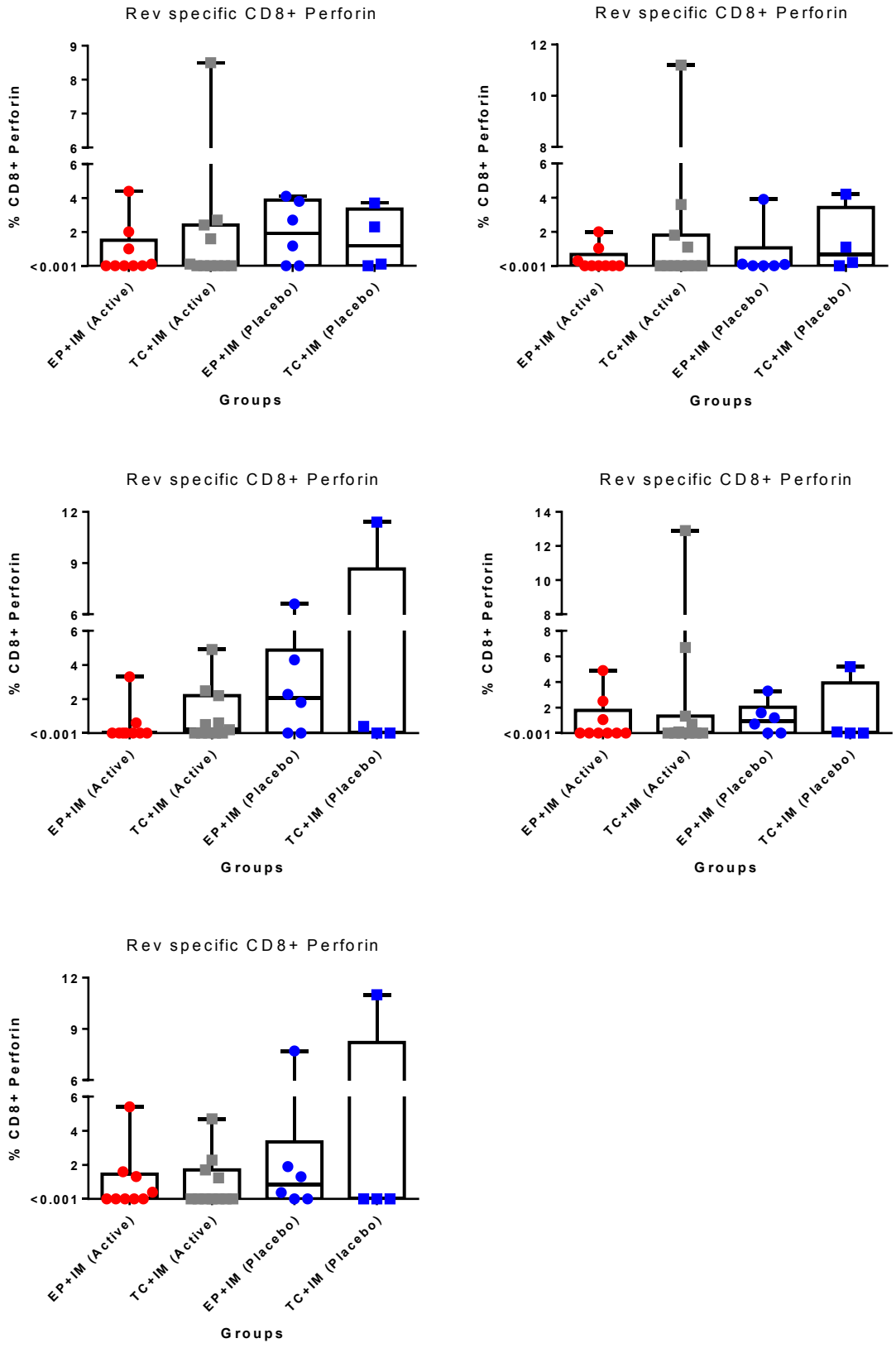


Figure 6.26: CD8+ perforin response at week 14 showing peptide specific responses comparing all groups. Statistical significance was set at p=<-0.05

Summary of Immunogenicity results: ICS

- CD4+ data: At week 14 responses were seen in the TC+IM (active) group which reached statistical significance compared to the EP+IM (active group) to IFN-ɣ and CD154
- CD8+ data: Similarly at week 14 responses were seen in the TC+IM (active) group to IFN-ɣ, TNF-α and CD154
- A comparison of week 0 and week 14 responses within each group showed a predominantly CD8+ response in the TC+IM group, specifically to IFN-ɣ, IL-2 and CD154

SECTION B: VIRAL INHIBITION ASSAY CUTHIVAC 001

6.4 INTRODUCTION

Having analysed the ELISpot and ICS data, the VIA was performed in order to assess CD8+ function as described previously. Samples were assessed from baseline (week 0) and the primary end point (week 14). In total 3 participant samples did not reach completion of the VIA due to sample contamination and are excluded from analysis: CT005 (EP+IM active), CT019 (TC+IM active), CT022 (EP+IM placebo).

As described in detail in the methods section (Chapter 2) in this viral inhibition assay autologous CD8+ and CD4+ cells were each expanded separately with their respective bispecific antibodies in order to evaluate anti-viral functions of CD8+ lymphocytes. Exogenous virus was then added to infect CD4+ cells and an objective method of p24 measurement using ELISA used to evaluate inhibitory activity.

The VIA in this trial was modified to take into account an HIV positive cohort with two main questions to be addressed. Full details can be found in the Chapter 2: Methods, however a brief summary is outlined below.

1. **Can CD8+ cells of vaccinated participants in the CUTHIVTHER trial inhibit endogenous virus production in an in vitro model?**

   This involved a modification of the VIA where CD4+ cells were not infected with exogenous virus as per standard assay, but p24 readings pre and post vaccination were analysed to answer whether endogenous virus was inhibited in this HIV positive cohort

2. **Can CD8+ cells of vaccinated participants in the trial in the CUTHIVTHER trial inhibit exogenous virus in an in vitro model?**

   To answer this question prior to CD4+ infection with exogenous virus, endogenous virus was blocked by adding maraviroc to the cultures, to ensure the final p24 readings were from exogenous virus replication. The assumption here was that endogenous virus was CCR5 tropic (and therefore a CCR5 inhibitor would block virus). A panel of 3 viruses was used for this stage; 1 clade B (IIIB) and 2 cross clade viruses (U455 Clade A and CBL4 clade D). All viruses were CXCR4 tropic, as the addition of the CCR5 inhibitor Maraviroc (see methods section) to the cultures would prevent the drug from blocking these X4 viruses added to cultures.

The modifications involved in setting up this assay have not been validated for an HIV positive cohort, therefore it is difficult to apply definitions to quantify a change in viral inhibition. From previous data available from chronic HIV infected individuals not on ART, levels of CD8+ mediated inhibition (measured by p24 in supernatant) can vary [244], therefore an assumption was made for this cohort on ART that there could be a level of viral inhibition at baseline and the pre-vaccination cut off of 0.6 log^{10} used in the preventative trial may be too low to detect changes in response. For the purposes of this assay the >1.5 log^{10} cut off for inhibition was applied, and general comments on changes in log^{10} inhibition are made.

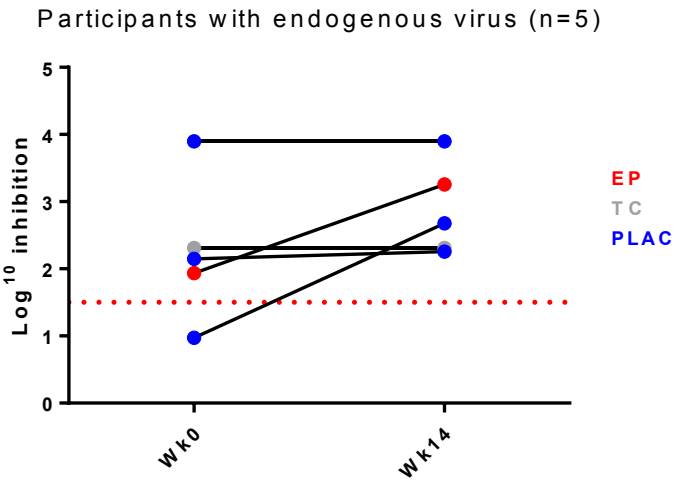
In this section the results from the VIA are presented to address the 2 questions outlined above, which is relevant to my second hypothesis and aim in HIV positive volunteers.

6.5 VIA RESULTS PART 1

Part 1 results: Can CD8+ cells of vaccinated participants in the CUTHIVTHER trial inhibit endogenous virus production in an in vitro model?

In this part of the VIA, endogenous viral replication pre-vaccination (week 0) and at the primary end point was compared (week 14), to determine if the CD8+ cells of vaccinated participants had an impact on endogenous viral replication. It was anticipated this cohort may have varying degrees of endogenous virus pre vaccination (week 0).

The results show at week 0, 5/30 (17%) participants had detectable levels of endogenous virus (measured by p24) (Figure 6.27). For these 5 participants with detectable endogenous virus, ELISA was conducted to determine p24 concentration as a measure of log^{10} inhibition and showed at week 0, 4/5 had log^{10} inhibition values of >1.5 suggesting these participants were inhibiting endogenous virus even at baseline. At week 14, in participants CT016 (EP+IM active) and CT002 (TC+IM placebo), these values had increased from baseline whereas in the other participants the log^{10} inhibition values remained the same (Table 6.8). The low number of participants with endogenous detectable p24 after stimulation, likely reflects the effectiveness of ART intervention and is discussed later. With small participant numbers it remains difficult to comment on whether vaccination has had an impact on the ability of CD8+ cells to inhibit endogenous virus, and of course one of these participants received placebo.



Participant ID	p24 (pg/ml)
CT016 (EP active)	11610
CT007 (TC active)	1545
CT009 (EP plac)	149440
CT002 (TC plac)	4972
CT012 (TC plac)	88460

Figure 6.27: Before-after plot to show the participants with detectable endogenous virus at week 0 (as measured by p24). Each dot joined by a line represents the log^{10} inhibition for one individual participant. The red dotted line is the cut off for positive inhibition of >1.5 log^{10}. The p24 values are shown in the table (pg/ml) for each responder.

Table 6.8: Endogenous virus production measured by p24 concentration in 5/30 participants at any time point with log^{10} inhibition shown at week 0 and week 14.

Participant	Group	Week 0 Log^{10} inhibition	Week 14 Log^{10} inhibition
CT016	EP+IM (active)	1.93	3.26
CT007	TC+IM (active)	2.31	2.31
CT009	EP+IM (placebo)	2.15	2.26
CT002	TC+IM (placebo)	0.97	2.68
CT012	TC+IM (placebo)	3.90	3.90

Summary of VIA results: Part 1

- 5/30 (17%) of participants had detectable levels of endogenous virus, and of these 4/5 displayed high levels of endogenous viral inhibition at week 0 (pre-vaccination)
- Of these only 2 participants showed a change in log^{10} inhibition at week 14, one of which had received placebo
- With small participant numbers it is difficult to draw concrete conclusions, but is it unlikely vaccination had an impact on the ability of CD8+ cells to inhibit endogenous virus in this cohort

6.6 VIA RESULTS PART 2

Part 2 results: Can CD8+ cells of vaccinated participants in the trial in the CUTHIVTHER trial inhibit exogenous virus in an in vitro model?

At week 0, 24/27 (89%) participants were found to have log^{10} inhibition values of >1.5 (inhibiting at least 1/3 viruses) suggesting in an HIV positive cohort on ART, there is some control of viral replication (mechanisms discussed later in this chapter). U455 (Clade A) virus was the most common virus to be inhibited pre-vaccination in 21/27 participants (78%), in line with data from CUTHIVAC 001 which showed a virus other than clade B was most commonly inhibited. At week 14, a total of 26/27 (96%) participants inhibited at least 1 virus with a more equal distribution of participants inhibiting each virus (Figure 6.28). Participants inhibiting virus at week 14 included participants in the placebo groups.

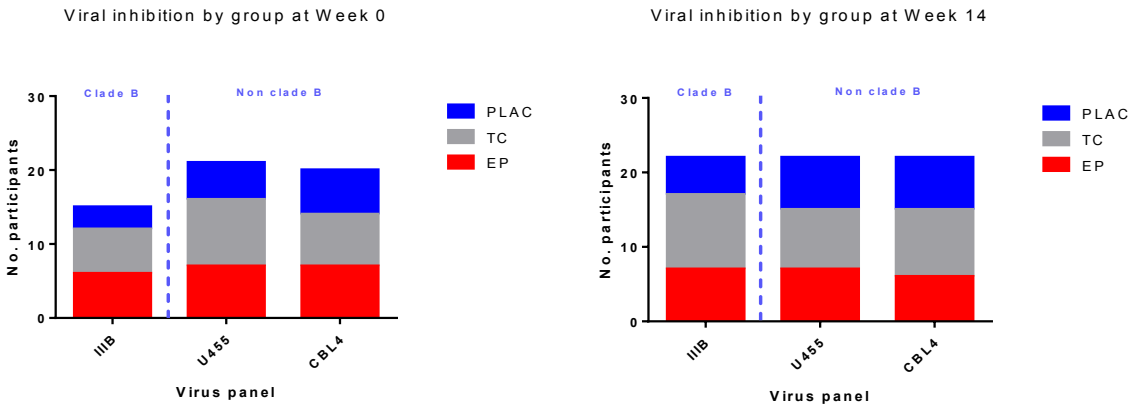
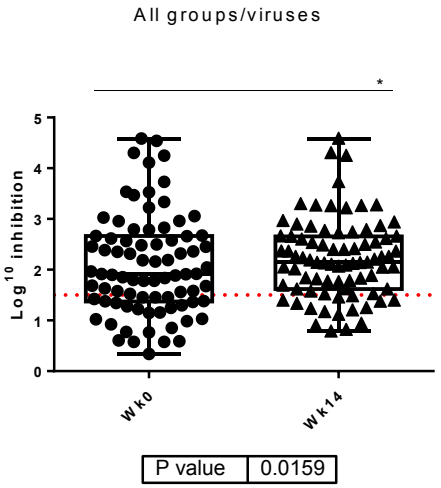
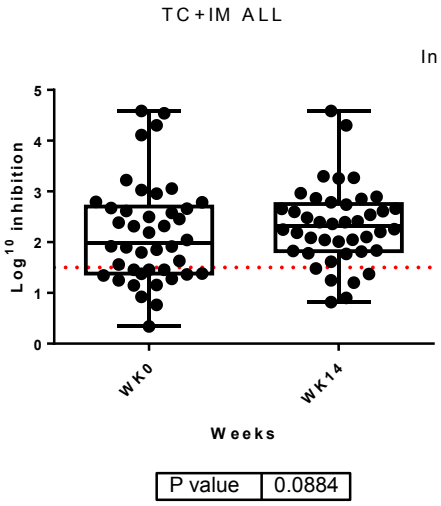


Figure 6.28: Bar charts to show the number of participants inhibiting each virus at week 0 and 14. The positive responders only are show by group.

Although a comparison of week 0 to week 14 for all groups and all viruses combined showed a trend towards enhanced viral inhibition at week 14 ($p=0.0159$), once this is broken down for each group, there were no significant differences for either group (TC+IM $p=0.884$, EP+IM $p=0.854$) (Figure 6.29).

There were no statistical differences in $\log^{10}$ inhibition between week 0 and week 14 found for any virus in the EP+IM (active) group, nor in the TC+IM (active) group, or in participants in either placebo groups (Figures 6.30-6.32). Similarly, there were no significant differences in $\log^{10}$ inhibition between the groups for any virus at week 14 (Figure 6.33).





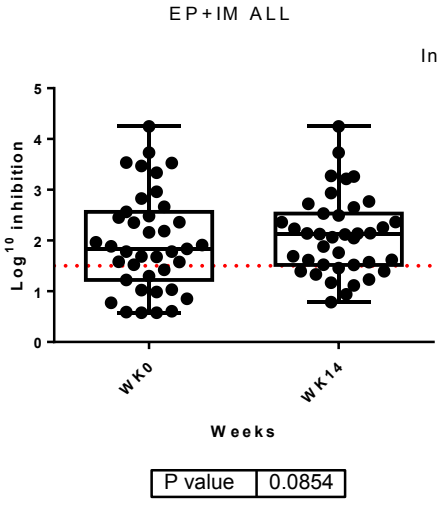
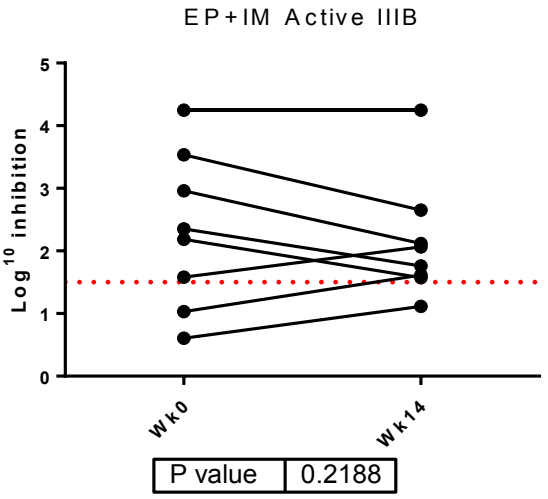
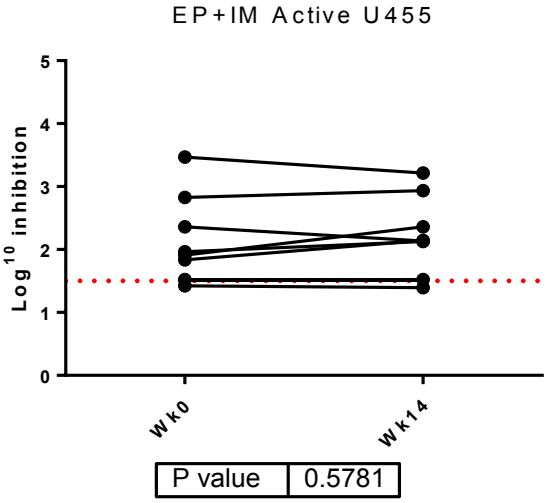


Figure 6.29: Graphs to show difference in log^{10} inhibition between week 0 and week 14 in all viruses and groups, the TC+IM group (including placebo) and the EP+IM group (including placebo). The red line represents the cut off for positive inhibition of >1.5 log^{10}. Statistical significance was set at p=<0.05.


EP+IM Active IIIB
Log10 inhibition
5
4
3
2
1
0
Wk0
Wk14
P value
0.2188


EP+IM Active U455
Log10 inhibition
5
4
3
2
1
0
Wk0
Wk14
P value
0.5781

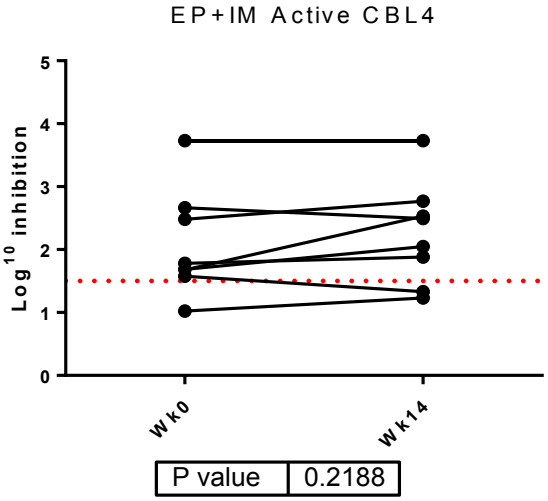

EP+IM Active CBL4
Log10 inhibition
5
4
3
2
1
0
Wk0
Wk14
P value
0.2188

Figure 6.30: Graphs for the EP+IM (active) group to show log^{10} inhibition for each virus at week 0 and week 14. Each dot joint by a line represents and individual participant's week 0 and week 14 values. P values are shown and statistical significance was set at p=<0.05.

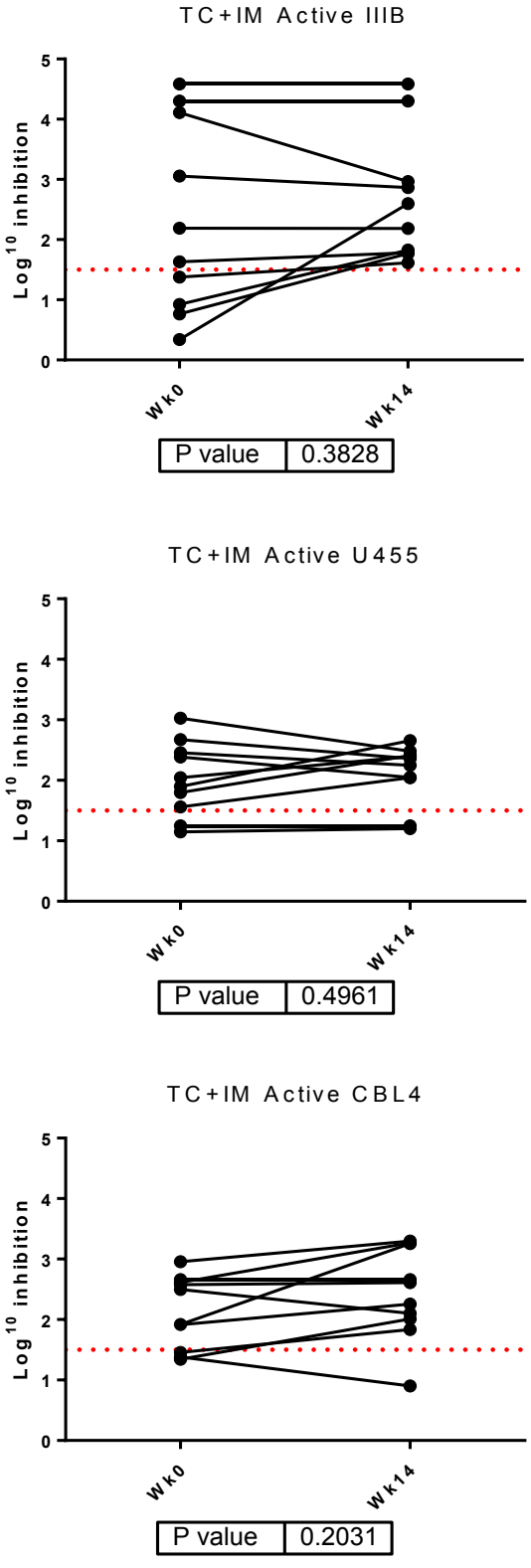


Figure 6.31: Graphs for the TC+IM (active) group to show log[10] inhibition for each virus at week 0 and week 14. Each dot joint by a line represents and individual participant's week 0 and week 14 values. P values are shown and statistical significance was set at p=<0.05.

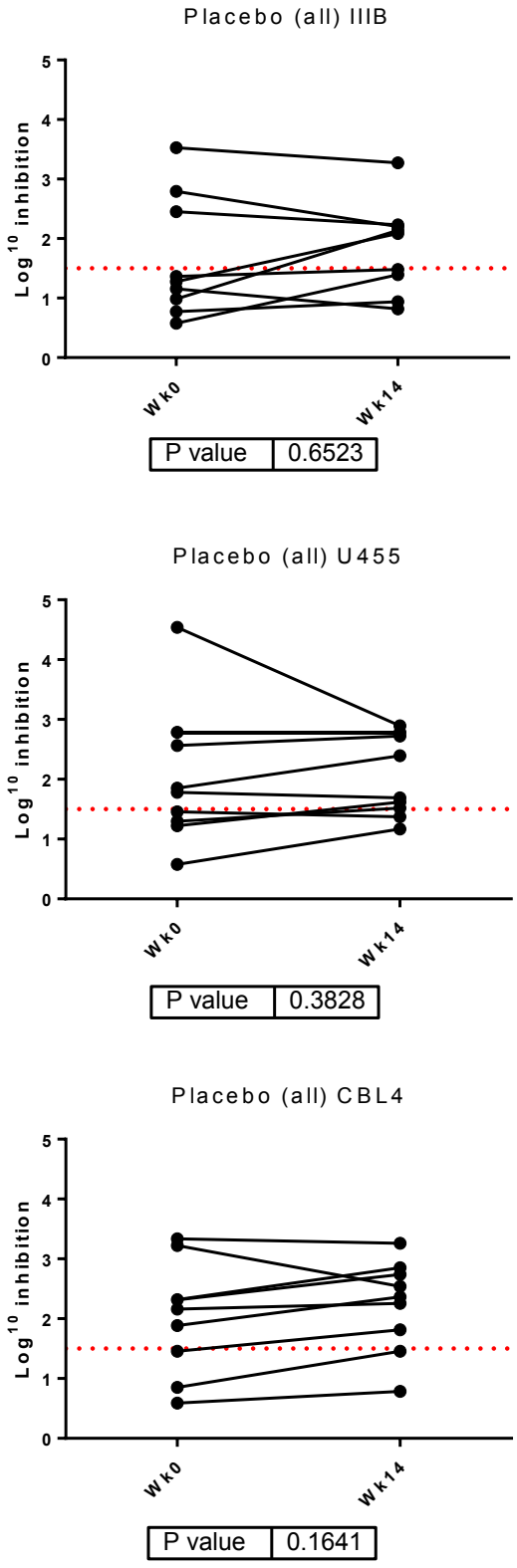


Figure 6.32: Graphs for the placebo group to show log^{10} inhibition for each virus at week 0 and week 14. Each dot joint by a line represents and individual participant's week 0 and week 14 values. P values are shown and statistical significance was set at p=<0.05.

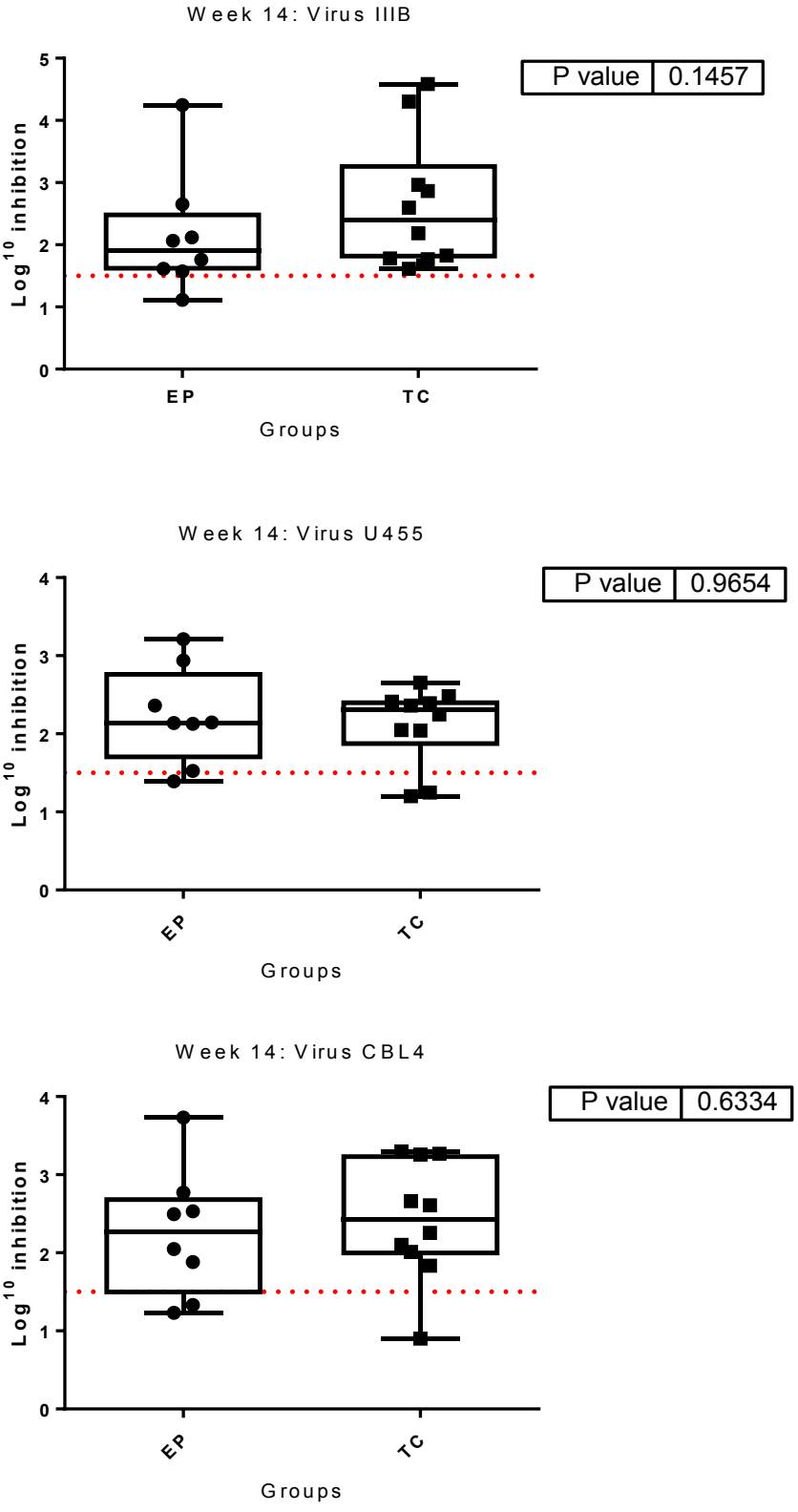


Figure 6.33: Box and whiskers graphs to show between group comparisons of log^{10} inhibition for each virus at week 14. Placebo recipients are included within their respective groups. P values are shown and statistical significance was set at p=<0.05.

Finally, with respect to breadth of viruses inhibited by each group at weeks 0 and 14, there was no increase in the median number of viruses inhibited in the EP+IM (active) group, but an increase was seen from 2 to 3 viruses in both the TC+IM (active) group and the placebo group (Figure 6.34 and Table 6.9). These differences however did not reach statistical significance. (p=0.5357).

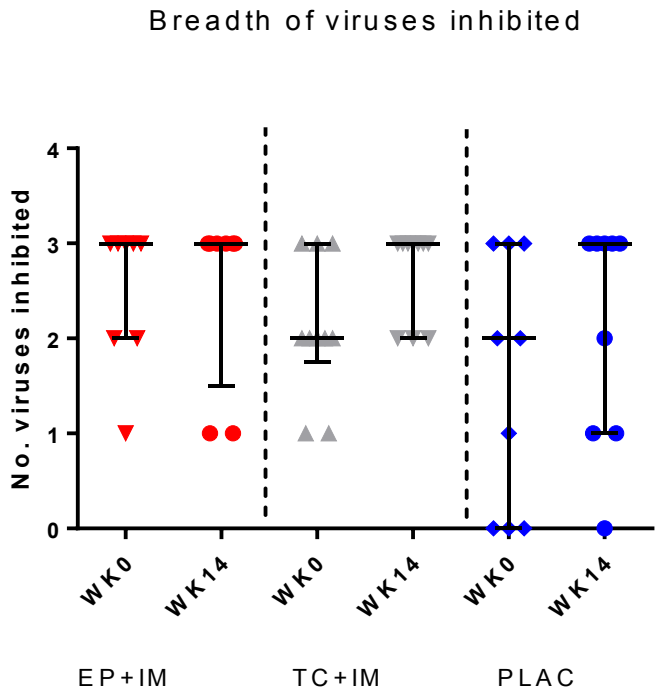


Figure 6.34: Breadth of viruses inhibited shown on graph at weeks 0 and 14 by group with median and interquartile range displayed.

Table 6.9: The table shows the median number of viruses inhibited by group at weeks 0 and 14.

Group	**Median number viruses inhibited**	
	Week 0	Week 14
EP+IM (active)	3.0	3.0
TC+IM (active)	2.0	3.0
Placebo (all)	2.0	3.0

Summary of VIA results: Part 2

- There was a general increase in viral inhibition at week 14 across pooled data from all groups, although this did not reach statistical significant when broken down by individual group
- There was a high level of viral inhibition at week 0 pre-vaccination , with U455 the most common virus inhibited
- There were significant differences in viral inhibition between the groups to any virus at week 14
- There was little change in median breadth of viruses inhibited at week 14 in any group

6.7 DISCUSSION

In the first section of this chapter I aimed to determine the immunogenicity of the GTU®MultiHIV B clade DNA vaccine using 2 DNA immunisation strategies (EP+IM and TC+IM), compared to placebo in an HIV positive cohort participating in the CUTHIVTHER 001 randomised phase I clinical trial. My aim was to describe both the phenotypic and functional T cell response for this trial, and to determine whether the T cell response is influenced by the route of DNA administration.

In this study, the IFN-ɣ responses at week 0 (pre vaccination) were of varying range and as expected many participants had high pre-existing responses to the peptide pools. However, overall there were no significant differences in the magnitude of IFN-ɣ at the primary end point between groups. With only a few responders in each group, it is unlikely vaccination via either regime had a detectable impact on immunogenicity. To summarise, neither active vaccination group had greater measurable HIV-specific immune responses than the other in terms of magnitude, breadth and duration of the IFN-ɣ response.

In addition, the variability of responses in the placebo group may indicate the definition of a 'positive' response of >2 fold increase was too lenient as this change was also seen in placebo recipients and therefore may not be stringent enough. The presence of changes in response from baseline in placebo recipients is likely to reflect the variability of background response to peptide pools in an HIV positive cohort, or the actual procedures themselves (electroporation and transcutaneous skin surface stripping) creating inflammation and activating cells in the absence of active drug. The observation of similar changes in the placebo group suggests changes in the active groups are likely non-specific and not related to active vaccination.

With respect to the integrase peptide pool, with so few responders it is unlikely that vaccination has resulted in non-specific immune changes in either group at the primary end point. The presence of responders to this peptide pool in placebo recipients raises similar questions of background variation or inflammation created by the vaccination process.

The use of electroporation in HIV positive participants has been shown to augment DNA vaccine delivery in one other recent HIV therapeutic vaccine trial where an interleukin-12 enhanced DNA vaccine delivered EP+IM to HIV positive participants on ART was shown to augment CD4+ but not CD8+ T cell responses using ICS [184]. In this trial 62 HIV positive participants on ART with low viral loads (<200 copies/ml) were randomized into receiving either vaccine or placebo and further randomised to receive HIV-MAG pDNA with increasing doses of IL-12 delivered IM+EP. Results

showed the vaccine induced small significant increases in CD4+T cell responses (by ICS) against multiple HIV antigens in the low dose DNA + MVA/IL-12 group.

To the best of my knowledge, this was the first trial using the transcutaneous route in combination with IM injection for DNA vaccination in an HIV positive cohort. In a previous trial using the same TC vaccine administration route (with CSSS), 6 HIV positive participants were vaccinated with Tetragrip (combined tetanus and influenza vaccine) and compared to the same vaccine delivered IM to 8 HIV positive patients. In the HIV positive volunteers receiving TC vaccination, there was a preferential amplification of vaccine-specific CD8+ cells (shown by ICS), with the authors concluding the results demonstrated this route of vaccination could affect the quality of the T cell response, albeit in small participant numbers [191].

In the CUTHIVTHER trial, a comparison of week 0 and week 14 ICS responses within each group showed a predominantly CD8+ response in the TC+IM group, specifically to IFN-ɣ, IL-2 and CD154, in keeping with the Tetragrip trial results. It remains important to add direct comparisons are difficult to make as the Tetragrip vaccine was not using DNA, nor TC vaccination in combination with IM. Although results from the ICS data were limited, those seen were predominantly in the TC+IM (active) group indicating the route of vaccination may well have an impact on the quality of the immune response and could not have been be predicted by IFN-ɣ ELISpot alone.

An interesting comparison can be made between the week 0 (pre-vaccination) IFN-ɣ response in this trial and the primary end point IFN-ɣ response in the CUTHIVAC (HIV uninfected cohort) trial. Pre vaccination responses in the CUTHIVTHER trial were of greater magnitude (particularly in *Nef* and *Gag* peptide pools) than post vaccination in the CUTHIVAC trial. However in this trial although pre-vaccination IFN-ɣ responses were high, there was little change post vaccination.

A potential factor to consider here is the dose of DNA was 50% less in this trial (2mg compared to 4mg), which may have had an impact on immunogenicity. The change in the protocol for this trial was based on CUTHIVAC 001 where the first participant receiving EP did not wish to carry on with the procedure due to pain. In hindsight, this decision to change the CUTHIVTHER protocol based on 1 participant may have been premature, as all other participants receiving IM vaccination with EP across both trials completed all vaccinations and deemed the procedure acceptable and tolerable. Whether this reduction in the dose of DNA may have affected the lack of change seen at the primary end point in this trial is difficult to determine but certainly a factor to consider when interpreting the immunogenicity results.

As stated previously it is not possible to make direct comparisons on change in viral load in this trial with the FIT-06 clinical trial in South African in infected Clade C participants not on ART, as all participants in CUTHIVTHER were on suppressive ART. However, changes in CD4+ counts seen in FIT-06 were not replicated in this trial. One factor to consider here is this cohort had been established on effective ART, some very early on in infection as part of the HEATHER cohort, therefore may have had effective restoration or near-restoration of immune function, making any significant changes in CD4+ count less likely.

In the second half of this chapter, I presented the VIA results assessing the functional CD8+ response. In Part A examining endogenous virus production, only 5 participants had detectable levels of endogenous virus measure by p24 ELISA. This is perhaps on the low side, but not entirely surprising as the classic ELISA is now no longer the most effective method to quantify p24 production. Newer techniques include ultrasensitive p24 assays, reported to be over 1000 times more sensitive than ELISA and able to detect viral proteins produced by a single infected cell [245].

It is not entirely clear why these particular 5 participants had detectable endogenous virus production compared to the other participants. Plausible reasons include limitations in the assay used to detect p24 which may have been insufficient to detect p24 in the majority of participants. Alternatively it may suggest in this particular cohort of healthy HIV positive people with relatively high nadir CD4+ counts and suppressive ART, there are sufficient functioning CD8+ mediated immune responses to control in vitro viral replication even prior to vaccination.

In Part B of the VIA assessing the ability of CD8+ cells to inhibit exogenous virus , participants in each group were able to inhibit the panel of viruses at week 0 again suggesting preservation of immune responses and specifically in this assay CD8+ function. There was little change post vaccination in ability to inhibit virus in any group, but participants shown to inhibit virus at week 14 also included placebo recipients. The modification of this assay needs further verification but could be a starting point for using the VIA in an HIV infected cohort.

Interestingly, at baseline it was the U455 virus (Clade A virus) which showed inhibition by the greatest number of participants, and not the clade-matched IIIB virus as expected. The VIA data from CUTHIVAC 001 (Results Chapter 4) also highlights a virus other than clade B was the most common virus to be inhibited, suggesting the vaccine may have some cross-clade activity.

There are several limitations that need to be taken into consideration. In terms of the trial, with such small participant numbers in each group it remains important not overstate the results, particularly for the VIA. In addition, the trial could have benefited from viral tropism data to guide use of

maraviroc in the VIA. Although those who were also enrolled in the HEATHER observational study were recently infected and likely to be R5 tropic, the remainder of participants may not have been R5 tropic. I was also not able to collect data on any changes in CD4+/CD8+ ratios. The modification of the protocol to receive EP+IM to one leg only (and therefore receive 2mg DNA only) may have also affected immunogenicity. Finally in hindsight, enrolling participants with relatively high CD4+ counts, no AIDS related complications and undetectable VLs, may have resulted in this cohort being 'too healthy' and therefore immunogenicity results may have been limited. Alternatively, this DNA vaccine may simply not have been immunogenic enough and could have benefitted from a vector boost as in the IL-12/MVA strategy.

Assay-related limitations include not being able to extend this project to include assessment on the size of the viral reservoir which may have given further insight into the impact of the vaccine. Finally, a period of analytical treatment interruption may have been useful for further information but was beyond the scope of this trial and not included in the protocol. In this particular trial T cell responses may not have been robust enough to justify treatment interruption.

In summary, neither IM DNA delivery with electroporation or delivered transcutaneously improved immunogenicity, as measured by IFN-ɣ and ICS, although I have shown there were high pre vaccination responses that were absent in HIV negative study participants. Similarly in the VIA there was no difference in inhibition between groups at the primary end point, although inhibition at baseline was shown in all groups (including placebo) suggesting in this healthy HIV positive cohort on ART, CD8+ function remains relatively preserved. Neither electroporation nor transcutaneous DNA delivery however had an impact on phenotypical or functional immunogenicity in this HIV positive cohort.

OVERALL SUMMARY OF IMMUNOGENICITY RESULTS FROM CUTHIVTHER 001

- IFN-ɣ T cell ELISpot results showed high pre-vaccination responses, with no significant differences in the magnitude, breadth or duration of response between groups at week 14
- There were responses seen in placebo recipients at week 14 suggesting high baseline variability in this cohort
- It is unlikely vaccination via either the TC or EP route in combination with IM resulted in non-specific immune changes as displayed in IFN-ɣ results from the integrase peptide pool
- A comparison of week 0 IFN-ɣ pre-vaccination responses in this trial to the primary end point response in the CUTHIVAC trial showed a significant difference in magnitude of the IFN-ɣ response at baseline in HIV infected volunteers
- Overall there was little change in CD4+ counts between week 0 and week 14 in any group
- The ICS data showed some CD4+ and CD8+ responses in the TC+IM (active) group, suggesting this route of vaccination can have an impact on the quality of the immune response
- There were high pre-vaccination CD8+ mediated viral inhibition of all viruses in all groups but no differences in inhibition at the primary end point
- Overall neither EP or TC delivery of DNA (in combination with IM) had a major impact on phenotypical or functional immunity in this HIV positive cohort

Chapter 7
Final Discussion

The work presented in this thesis aimed to characterise the T cell response in HIV negative and HIV positive participants (on ART). Participants in both trials were immunised with the GTU®MultiHIV B clade DNA vaccine to determine whether the T cell response was influenced by the route of vaccine administration, and to evaluate the functionality of HIV-specific T cell responses using a viral inhibition assay in these two cohorts.

Summary of results: CUTHIVAC 001 preventative vaccine trial

In my primary hypothesis I speculated that DNA vaccination could induce HIV-specific T cell immunity in an HIV negative cohort and induce antiviral function *in vitro* demonstrated in the VIA. Addressing my first hypothesis I showed in the HIV negative cohort DNA vaccination did induce HIV-specific T cell immunity specifically in the group receiving EP+IM vaccination, showing a greater magnitude, breadth and duration of response across all peptide pools by IFN-ɣ at the primary end point.

Addressing my second hypothesis, through the VIA I was able to show a degree of functionality of the induced HIV-specific immune responses, in this cohort as evidenced by $\log^{10}$ inhibition as a marker of CD8+ protection *in vitro*. Although the EP+IM group demonstrated the greatest CD8+ mediated inhibition in the most participants, both TC+IM and ID+IM groups also showed inhibition of viral replication despite limited immunogenicity by IFN-ɣ ELISpots.

Summary of results: CUTHIVTHER 001 therapeutic vaccine trial

Addressing my primary hypothesis in the HIV positive cohort I showed although there were high pre-vaccination responses, neither TC+IM or EP+IM groups had significant changes in the IFN-ɣ response either comparing each group or to placebo. Therefore in this cohort, the vaccine did not induce additional HIV-specific immunity as measured by IFN-ɣ with either route of vaccine administration.

Addressing my second hypothesis in this cohort the VIA was modified to demonstrate inhibition of both endogenous and exogenous virus. With respect to endogenous viral inhibition, results showed it was unlikely either route of vaccination had an impact on the ability of CD8+ cells to inhibit endogenous virus production from activated T-cells in vitro. Similarly using a panel of three exogenous viruses, although there was a high level of viral inhibition seen pre-vaccination across all

participants, there were no significant differences in viral inhibition between groups to any virus at the primary end point, nor in comparison to the placebo group. Therefore in this cohort DNA vaccination did not enhance baseline viral inhibition.

Questions remaining unanswered

In addressing these hypotheses, a number of questions remain unanswered. In both trials, there was a discrepancy between the IFN-ɣ ELISpot assay results and the VIA results, particularly in the HIV negative cohort where participants in the TC+IM group showed inhibition despite limited immunogenicity by IFN-ɣ ELISpot. This raises questions as to which assay or combination of assays should be used in future vaccine trials to address all aspects of immunogenicity. IFN-ɣ ELISpots enable direct visualisation of single reactive cells, providing a relatively simple method of quantifying vaccine responses. However this is only a single cytokine response, and whilst this method is highly reproducible across vaccine trials, there is limited data on whether IFN-ɣ is an adequate or accurate reflection of antiviral function [231].

Both trials and cohorts showed greater viral inhibition to the non-Clade B viruses. With limited data on the exact vaccine construct of the GTU®MultiHIV B clade DNA vaccine it is difficult to know the significance of this and whether this may have clinical implications.

It is perhaps the therapeutic trial that has raised more questions than answered. The immunogenicity results across all assays used in this trial were limited and modest in comparison to the preventative trial. Reasons for this have already been suggested (Chapter 6), but point to considerations on the immune background or status of participants enrolled and whether in this particular trial participants were simply too healthy as reflected by relatively high nadir CD4 counts, and control of viraemia on ART. The presence of immunological responses seen in placebo recipients is also intriguing and further points to a lack of HIV-specific T cell responses seen in this trial.

Utility of electroporation in HIV vaccine trials compared with prime-boost regimens

In the context of a preventative HIV vaccine trial the GTU®MultiHIV B clade DNA vaccine showed comparable results primarily in the EP+IM group in terms of magnitude, breadth and duration of the IFN-ɣ response comparable to other clinical trials using EP. Should this DNA vaccine be used in a prime boost regimen, perhaps even greater immunogenicity results would be seen. In general, DNA prime-boost approaches focus on the induction of T cell responses, and viral vectors used as a

'boost' have in the past included adenovirus, vaccinia, and vesicular stomatitis virus [246]. DNA priming has been shown to improve the frequency, strength and breadth of anti-HIV CD4+ and CD8+ T cell responses and to improve persistence of these responses, even when initially no T cell responses are detected after the DNA prime [247]. In particular, the modified vaccinia virus Ankara (MVA) has shown the most promise when used as a DNA boost [248], with both T cell and ADCC mediated Ab responses demonstrated in a recent clinical trial using DNA in Tanzanian HIV uninfected volunteers [249].

With effective prime boost regimens the question then arises of whether other methods of enhancing DNA immunogenicity are required, and in the context of this thesis whether there is a need for EP with an effective prime-boost regimen using DNA. The applied electric field during electroporation disrupts cell membrane structures creating a temporary membrane instability and allowing previously impenetrable substances (such as DNA) to readily cross the cell membrane [226]. The demonstration in preclinical studies that EP could dramatically increase DNA uptake in skin and muscle in comparison to DNA delivered through conventional IM injection initially provided the drive for EP to enhance delivery against a wide range of disease indications [250].

The use of EP has now been investigated in over 10 clinical trials across a variety of diseases, with all studies designed with the primary objective of evaluating safety and tolerability of this vaccine approach. The majority of studies have been based on IM delivery of the vaccine with EP, with newer devices focusing on ID delivery with EP in the context of HIV [183]. A clinical trial comparing DNA delivery and EP with a DNA prime-boost regimen may address the question of the utility of EP in this context. Such a trial is yet to be conducted.

The use of EP in therapeutic HIV vaccine trials is limited with only one trial reporting to date on the use of DNA delivered with EP for the treatment of HIV infection [184]. In this trial HIV-1 MAG/DNA in combination with IL-12 was delivered by IM-EP and was shown to augment CD4+ but not CD8+ T cell responses to multiple HIV antigens in HIV positive participants on ART. These CD4+ T cell responses were deemed to be of potential clinical interest by the authors, as CD4+ T cells provide important support for CD8+ T cell function and memory formation [251]. In addition in therapeutic vaccine trials CD4+ responses have been shown to be associated with control of viraemia in analytical treatment interruption [252]. In my therapeutic vaccine trial all responses were limited, suggesting newer vaccine strategies are needed in the context of therapeutic vaccine trials. However, it cannot be excluded that the vaccine may have primed cells that could have been boosted if combined with a vector such as MVA and or adenovirus.

Newer therapeutic vaccine trial strategies

One such vaccine strategy is the development of a novel conserved-region T cell mosaic vaccine. Arguably one of the greatest challenges in the development of an effective vaccine against HIV-1 surrounds the genetic diversity of the virus. By targeting highly conserved regions of HIV-1 proteins this genetic diversity may be overcome as mutations in these regions are more likely to cause a fitness loss [253],[254]. Furthermore, immunogens are now in development using highly conserved regions of HIV-1 proteins assembled into a chimeric immunogen HIVconsv, which when delivered combined with plasmid DNA, simian adenovirus (ChAdv) and MVA, have been shown to induced HIV-specific T cells capable of inhibiting replication in CD4+ cells *in vitro* [255],[256].

Although the applicability *in vivo* has yet to be determined, recent data has been presented at the Conference on Retroviruses and Opportunistic infections (CROI 2017) from the BCN02 trial using a similar HIVconsv vaccine. In this as yet unpublished data 15 HIV positive participants who had started early treatment after infection were given 3 doses of the HIVconsv vaccine following by 3 infusions of the latency reversing drug romidepsin [257]. HIV-specific CD8+ responses were found to have increased during the study after the vaccination process and romidepsin. 13 participants entered a treatment interruption phase where ART was stopped; in 8 the viral load rebounded rapidly, however the remaining 5 participants stayed off ART maintaining an undetectable VL for between 6 to 28 weeks. The trial has not yet concluded its report but represents a promising first step in demonstrating post-treatment control using the novel HIVconsv vaccine.

Applicability of DNA delivery techniques

With respect to the applicability and use of the three vaccination modalities in future clinical DNA HIV vaccine trials, it is the ID+IM procedure used in the preventative trial (CUTHIVAC 001) which showed the most limited results and hence was not taken forward to the therapeutic trial. Although perhaps not adequately immunogenic in this trial, in general there are several advantages to intradermal vaccination including dose reduction compared to IM, simplicity of delivery and the ability to standardize ID delivery with newer devices [258]. My results may have showed limited immunogenicity with DNA and IM vaccination in this research thesis, but it should not be underestimated as ID vaccination in a prime-boost model or in combination with EP has shown promising results in both pre-clinical and clinical trials [183].

In a recent Phase IIa randomised clinical trial intradermal priming with HIV-1 DNA followed by MVA boosting in HIV negative volunteers based in Tanzania aimed to examine whether the use of ID

vaccination as a prime could reduce the number of priming injections needed which could have important feasibility implications in the developing world. In this clinical trial the HIV-DNA vaccine consisted of 7 plasmids carrying different HIV-1 genes delivered either via 2 intradermal doses of combined DNA plasmid pools, 2 ID injections of separated plasmid pools or the 'standard' 5 ID injections of separated plasmid pools [259]. Results showed the simplified regimen of 2 intradermal injections of the combined plasmid pools had a similar priming effect as the standard regimen of 5 intradermal injections, an important factor in planning for larger clinical trials and highlighting the utility of intradermal vaccination as a primer.

The transcutaneous method in combination with IM DNA vaccine delivery showed a different qualitative pattern of immune response. Considerations of real world applicability must take into account the feasibility of delivering such a method of vaccine delivery. As explained in detail in the methods section (Chapter 2), this technique is currently lengthy involving a 20 minute glue application procedure to disrupt the stratum corneum, followed by a further 20 minutes of absorption once the vaccine is applied requiring participants to stay in clinic with their arm elevated to aid absorption. Furthermore, with evidence of hypo and hyperpigmentation changes particularly in black and other darker skin combinations, currently this route of vaccine delivery may not be ideal in the developing world.

With DNA electroporation, as outlined previously the benefits of this mode of vaccine delivery to enhance DNA immunogenicity has now been established across several pre-clinical and clinical trials. However, as with transcutaneous immunisation the real world application of EP has to be taken into consideration. Currently safety and tolerability is acceptable with EP, yet the procedure is still invasive and associated with pain and discomfort. Current devices are simply not feasible for mass roll-out vaccination campaigns. However, manufacturers are looking to simplify devices with battery-operated systems currently in the design stage.

Advances in DNA delivery technology

As referenced previously the ID+EP device has already been used in the context of HIV-1 preventative trials and found to be immunogenic [183]. The development of newer ID+EP devices includes a multi head delivery device which may be able to overcome the volume restrictions of single ID vaccinations [260]. Such a device will enable simultaneous ID delivery to multiple sites and increase the number of cells transfected which is postulated to have a significant impact on the immune response generated in clinical trials. Currently this device is being investigated pre-clinically until more results become available.

The importance of the skin as a vaccine target however cannot be ignored. Newer methods of improving transcutaneous delivery are in progress. Sonophoresis refers to the use of ultrasound to deliver vaccines across the dermis [261], where the use of ultrasound creates cavitation or pores in the skin improving and aiding skin permeability. The size of the skin pores created can be varied by controlling the frequency and intensity of ultrasound [262]. Preclinical studies using this technique in mice to aid transcutaneous delivery of the tetanus toxoid have shown low dose ultrasound exposure followed by immunization with tetanus toxoid led to significant levels of anti-tetanus toxoid IgG with protective neutralising antibodies when compared to transcutaneous delivery without ultrasound, although responses were lower than in conventional IM vaccination [263].

Another technique used to aid transcutaneous skin delivery is iontophoresis which refers to the use of a mild electric current applied through small conduction electrodes in contact with the skin. This results in ions migrating through the skin towards the electrode of opposite charge, facilitating the transport of neutral and positively charged molecules across the skin [264]. So far preclinical trials investigating drug targets have shown some promise but in the context of vaccines, data is fairly limited [265]. Interestingly this technique has been reported to be pain free and non-invasive, compared to the more physical method of EP [266]. Most recently pre-clinical trials have examined the use of iontophoresis in combination with nanoparticles (described below) for transcutaneous immunisation. Here when the low electrical current used in iontophoresis was applied using ovalbumin as the antigen in combination with silver nanoparticles, penetration into the epidermis was increased by over 90-fold in comparison to passive delivery [267].

As stated previously, the main challenge with TC vaccination is overcoming the stratum corneum to increase antigen permeability. Much interest has been focused on the investigation of small molecules such as nanoparticles (NPs) or liposomes to enhance TC vaccine delivery. NPs are solid colloidal particles ranging in size from 1 to 1000nm, and can be used both as potential drug delivery vectors across the skin and to safely and effectively deliver DNA into cells [268]. NPs interact with skin lipids inducing transient and reversible permeability of the stratum corneum, thus enhancing TC delivery. In addition, NPs are thought to be able to penetrate hair follicles where there are a high number of APCs, therefore have the ability to enhance immune responses [269]. NPs have been used in prime-boost regimens utilising DNA in pre-clinical trials, and in the context of HIV have been shown to elicit HIV-specific humoral and T cell response to *Tat* when delivered using nanoparticles in mice [270].

Combining transcutaneous immunisation with liposomes is also currently under investigation. Liposomes are small lipid molecules (usually less then 10nm in size) which form due to hydrophobic and hydrophilic lipid interactions, and allow encapsulation of DNA for efficient delivery [171]. Although on their own liposomes are impenetrable to the stratum corneum, when combined with activators such as surfactants, the liposomes are able to squeeze through lipid channels most likely through the process of osmosis [271]. Pre-clinical trials have highlighted the use of liposomes to aid TC vaccine delivery against hepatitis B, and tetanus toxoid [272],[273].

Finally, patch based vaccination has been one of the most widely used systems for effective TC delivery. Pre-clinical trials have shown the use of a transcutaneous immunostimulatory patch for influenza placed on the skin in mice at the site of DNA influenza vaccine injection (IM), amplified anti-influenza antibody immune responses [274], and similar results using a patch have been seen with other diseases including tetanus and diphtheria [275]. More recently a 'nanopatch' has been developed and is currently undergoing pre-clinical trials. This nanopatch consists of a small patch with short microprojections (approximately 100μm each) with the ability to deliver vaccine antigen to thousands of skin APCs in the dermis and epidermis [276]. In pre-clinical trials used with the influenza vaccine in mice, use of the nanopatch resulted in a decrease in the amount of influenza antigen required to produce an antibody response by 1/100 compared to IM vaccination, with data also available on practicality, heat stability and utility in low resource settings [277]. Although it is clear many challenges still exist in improving and enhancing TC immunisation, this route of vaccine administration provides a novel and attractive avenue for future clinical trials. Newer EP devices as mentioned include the intradermal EP device, and more recently the development of a multielectrode array device. This device could overcome dose/volume restrictions, allow multiple sites to be treated simultaneously and potentially deliver a combination of antigens [260]. On a global scale this may be clinically relevant in the context of emerging infectious diseases or pandemics such as Ebola or malaria, and of course in combination with DNA vaccination could be cost effective and easier to mass produce than other vaccine modalities. However, much research is still needed on feasibility and real world implications, as well as the development of more user and patient friendly devices.

The future of DNA vaccination

The use of DNA for vaccination against a variety of diseases remains a work in progress, with several advantages already mentioned including cost-effectiveness, relative ease of mass production and heat stability. Whilst a wide variety of strategies have been developed to optimize DNA vaccine immunogenicity, the fact remains immunogenicity in humans is limited by low levels of antigen expression when compared to more conventional protein vaccines and currently newer general approaches are needed if human DNA vaccines are to be licensed in the near future.

A 'systems biology' approach is currently in progress and may provide more promise for the future of DNA vaccination. Here genetic sequencing, advanced bioinformatics analysis and 'omics' are utilised to develop new DNA vaccines. Omics refers to the combination of modern immunological techniques, existing knowledge, computational modelling and immunogenetics to fully characterise the host immune response and is regarded as the next step forward in DNA vaccination techniques [278],[279]. With such an approach or combination of approaches, there is potential for human DNA vaccination to emerge as a likely candidate for successful HIV vaccine delivery, although this is more likely to be in the context of HIV prevention rather than therapeutics.

However, the future may lie not with DNA but with RNA vaccination. RNA vaccines have already shown adaptability and advantages over DNA vaccination. Importantly, RNA vaccines are directly translated in the cytoplasm. Therefore they do not need to be delivered into the cell nucleus post injection as with DNA [280], nor require the use of electroporation. In addition, in preclinical trials RNA vaccines have been shown to induce both humoral and cell mediated immunity, be safe, robust and also rapidly manufactured [281]. In clinical trials RNA vaccines have been utilised in cancer patients supported by preclinical animal model data [282], with other similar cancer clinical trials in progress. Whether these early trials can be translated to HIV vaccination is yet to be determined.

Turning considerations to HIV vaccination in the context of HIV prevention, since HIV was formally identified as the cause of AIDS in the early 1980s, there have been ongoing efforts to develop preventative HIV vaccines now spanning over 3 decades. With no likely vaccine candidate currently emerging , several questions arising from clinical trials remain unanswered. Most recently with preliminary unpublished data in the UK (and particularly London) showing up to a 40% drop in new HIV diagnoses, the question arises of whether a preventative vaccine is still needed to change the curve of the epidemic. This substantial drop in HIV diagnoses has mainly been attributable to a combination of the uptake of PrEP in at risk populations in the UK, and early initiation of ART for all

(treatment as prevention). Therefore if such a dramatic decrease in new infections continues, is there still a need for a preventative vaccine against HIV?

In addressing this it remains important to consider the cost of ART and PrEP and the availability and ability to roll these treatments out to countries with the highest prevalence of HIV, which already cannot keep up with current ART costs. Several projected mathematical models still predict a vaccine remains one of the most cost effective ways of HIV prevention, particularly in lesser economically developed countries [283],[284]. However, it is likely the future of HIV prevention lies in a combination of prevention measures [285]. For now the addition of a preventative vaccine remains elusive.

Future prospects of a functional cure may be realised with improving knowledge on the HIV reservoir and an available ideal model to work from in the form of elite controllers of HIV. As demonstrated in early data from the BCN02 trial presented at CROI and other similar therapeutic vaccine trials currently in progress, this could result in viral control off ART when a vaccine is combined with a latency reversing agent. Again cost considerations are also important here, particularly in the context of novel expensive immunotherapeutic agents and global availability of these therapeutic interventions [136]. However, the projected costs of treating people living with HIV globally may well be offset should there be a successfully approach to a functional cure in the near future.

My future work

The question remains to whether the GTU®MultiHIV B clade DNA vaccine should be included in future clinical trials. Based on the results from the first preventative trial, a second study (CUTHIVAC 002) is already underway. This is a phase I study aimed at exploring the safety and immunogenicity of 2 different modes of delivering a DNA vaccination (DNA-C CN54ENV) via combined IM and ID methods with and without EP and boosted with recombinant HIV CN54gp140 administered by ID injection (clinicaltrials.gov). Although this is not using the same DNA vaccine as in this work, the combinations of ID/EP and IM/EP and the use of a protein boost will be interesting options to explore and give additional data on delivery methods using DNA and EP.

Using the GTU®MultiHIV B clade DNA vaccine the next step in terms of future work could be to address the question of whether EP is needed to enhance DNA delivery if the GTU®MultiHIV B clade DNA vaccine is combined with a vector or protein boost. In HIV negative volunteers the safety and immunogenicity of the vaccine delivered EP+IM group could be directly compared with the DNA

vaccine in a prime-boost regimen. In the context of therapeutics, it would also be important to assess whether this vaccine could elicit immunogenicity when used in a prime-boost regime. However, in an era where treatment is recommended for everyone and with previous data from the SMART study showing episodic ART carries substantial risks [286], any form of analytical treatment interruption would have to take place with intensive monitoring and ethical considerations.

It would have been interesting in the therapeutic trial to have been able to assess whether there were any changes in the HIV reservoir in vaccinated participants. This would have required the use of assays to measure integrated full length, functional HIV DNA in resting CD4+ subsets, and include PCR techniques to measure HIV integration in PBMCs [287].

In conclusion, this thesis has highlighted the T cell response in two very different cohorts using the same DNA vaccine. My aims and hypothesis have been solely based on the magnitude, quality and function of the T cell response, but of course the most comprehensive HIV vaccine strategy will likely involve the ability to induce a neutralizing antibody response in addition to robust HIV-specific CD8+ T cell responses. Most future studies therefore should be focused on developing neutralising antibodies or T cell responses separately before combining studies into one vaccine field. For the immediate future, the role of DNA vaccination despite displaying many advantages in the development of a preventative or therapeutic vaccine, remains uncertain.

References

1. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, et al. **Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency**. *N Engl J Med* 1981; 305(24):1425-1431.
2. **Pneumocystis pneumonia--Los Angeles**. *MMWR Morb Mortal Wkly Rep* 1981; 30(21):250-252.
3. Masur H, Michelis MA, Greene JB, Onorato I, Stouwe RA, Holzman RS, et al. **An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction**. *N Engl J Med* 1981; 305(24):1431-1438.
4. Gallo RC, Montagnier L. **The discovery of HIV as the cause of AIDS**. *N Engl J Med* 2003; 349(24):2283-2285.
5. Goedert JJ, Gallo RC. **Epidemiological evidence that HTLV-III is the AIDS agent**. *Eur J Epidemiol* 1985; 1(3):155-159.
6. Gallo RC. **HIV--the cause of AIDS: an overview on its biology, mechanisms of disease induction, and our attempts to control it**. *J Acquir Immune Defic Syndr* 1988; 1(6):521-535.
7. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, et al. **The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial**. *N Engl J Med* 1987; 317(4):185-191.
8. Broder S. **The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic**. *Antiviral Res* 2010; 85(1):1-18.
9. **UNAIDS 2014 Global Statistics. www.unaids.org**. In; 2014.
10. WHO. **HIV/AIDS Factsheet 360**. In; 2016.
11. PHE. **HIV in the UK: 2016 Report**. In; 2016.
12. Sharp PM, Hahn BH. **Origins of HIV and the AIDS pandemic**. *Cold Spring Harb Perspect Med* 2011; 1(1):a006841.
13. Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, et al. **Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes**. *Nature* 1999; 397(6718):436-441.
14. Keele BF, Van Heuverswyn F, Li Y, Bailes E, Takehisa J, Santiago ML, et al. **Chimpanzee reservoirs of pandemic and nonpandemic HIV-1**. *Science* 2006; 313(5786):523-526.
15. Pepin J. **The origins of AIDS: from patient zero to ground zero**. In: *J Epidemiol Community Health*. England; 2013. pp. 473-475.
16. Pepin J, Labbe AC, Mamadou-Yaya F, Mbelesso P, Mbadingai S, Deslandes S, et al. **Iatrogenic transmission of human T cell lymphotropic virus type 1 and hepatitis C virus through parenteral treatment and chemoprophylaxis of sleeping sickness in colonial Equatorial Africa**. *Clin Infect Dis* 2010; 51(7):777-784.
17. Pepin J, Lavoie M, Pybus OG, Pouillot R, Foupouapouognigni Y, Rousset D, et al. **Risk factors for hepatitis C virus transmission in colonial Cameroon**. *Clin Infect Dis* 2010; 51(7):768-776.
18. Bhattacharya T, Daniels M, Heckerman D, Foley B, Frahm N, Kadie C, et al. **Founder effects in the assessment of HIV polymorphisms and HLA allele associations**. *Science* 2007; 315(5818):1583-1586.
19. Buonaguro L, Tornesello ML, Buonaguro FM. **Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications**. *J Virol* 2007; 81(19):10209-10219.
20. Durand S, Cimarelli A. **The inside out of lentiviral vectors**. *Viruses* 2011; 3(2):132-159.
21. Sierra S, Kupfer B, Kaiser R. **Basics of the virology of HIV-1 and its replication**. *J Clin Virol* 2005; 34(4):233-244.
22. Chan DC, Fass D, Berger JM, Kim PS. **Core structure of gp41 from the HIV envelope glycoprotein**. *Cell* 1997; 89(2):263-273.
23. Gomez C, Hope TJ. **The ins and outs of HIV replication**. *Cell Microbiol* 2005; 7(5):621-626.
24. Nielsen MH, Pedersen FS, Kjems J. **Molecular strategies to inhibit HIV-1 replication**. *Retrovirology* 2005; 2:10.

25. Barre-Sinoussi F, Ross AL, Delfraissy JF. **Past, present and future: 30 years of HIV research**. *Nat Rev Microbiol* 2013; 11(12):877-883.
26. BASHH. **UK guideline for the use of HIV post-exposure prophylaxis following sexual exposure (PEPSE) 2015.** In; 2015.
27. **Public Health England. HIV new diagnoses, treatment and care in the UK. 2015 Report.** In.
28. Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, Peddada L, et al. **Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection**. *Aids* 2003; 17(13):1871-1879.
29. Cohen MS, Gay CL, Busch MP, Hecht FM. **The detection of acute HIV infection**. *J Infect Dis* 2010; 202 Suppl 2:S270-277.
30. Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, et al. **A whole-genome association study of major determinants for host control of HIV-1**. *Science* 2007; 317(5840):944-947.
31. Fellay J, Ge D, Shianna KV, Colombo S, Ledergerber B, Cirulli ET, et al. **Common genetic variation and the control of HIV-1 in humans**. *PLoS Genet* 2009; 5(12):e1000791.
32. Abbas A K LAH. **Basic Immunology: functions and disorders of the immune system**. 2004.
33. Borrow P. **Innate immunity in acute HIV-1 infection**. *Curr Opin HIV AIDS* 2011; 6(5):353-363.
34. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, et al. **DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells**. *Cell* 2000; 100(5):587-597.
35. Tsunetsugu-Yokota Y, Yasuda S, Sugimoto A, Yagi T, Azuma M, Yagita H, et al. **Efficient virus transmission from dendritic cells to CD4+ T cells in response to antigen depends on close contact through adhesion molecules**. *Virology* 1997; 239(2):259-268.
36. McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. **The immune response during acute HIV-1 infection: clues for vaccine development**. *Nat Rev Immunol* 2010; 10(1):11-23.
37. Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, Heitman J, et al. **Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections**. *J Virol* 2009; 83(8):3719-3733.
38. Jost S, Altfeld M. **Control of human viral infections by natural killer cells**. *Annu Rev Immunol* 2013; 31:163-194.
39. Bashirova AA, Thomas R, Carrington M. **HLA/KIR restraint of HIV: surviving the fittest**. *Annu Rev Immunol* 2011; 29:295-317.
40. Altfeld M, Gale M, Jr. **Innate immunity against HIV-1 infection**. *Nat Immunol* 2015; 16(6):554-562.
41. Mocikat R, Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U, et al. **Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses**. *Immunity* 2003; 19(4):561-569.
42. Hall A YC. **Immunology**. 2010.
43. Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA, et al. **The antigenic structure of the HIV gp120 envelope glycoprotein**. *Nature* 1998; 393(6686):705-711.
44. Burton DR, Mascola JR. **Antibody responses to envelope glycoproteins in HIV-1 infection**. *Nat Immunol* 2015; 16(6):571-576.
45. Smalls-Mantey A, Doria-Rose N, Klein R, Patamawenu A, Migueles SA, Ko SY, et al. **Antibody-dependent cellular cytotoxicity against primary HIV-infected CD4+ T cells is directly associated with the magnitude of surface IgG binding**. *J Virol* 2012; 86(16):8672-8680.
46. Forthal DN, Moog C. **Fc receptor-mediated antiviral antibodies**. *Curr Opin HIV AIDS* 2009; 4(5):388-393.
47. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. **Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand**. *N Engl J Med* 2009; 361(23):2209-2220.

48. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. **Immune-correlates analysis of an HIV-1 vaccine efficacy trial**. *N Engl J Med* 2012; 366(14):1275-1286.
49. Sather DN, Armann J, Ching LK, Mavrantoni A, Sellhorn G, Caldwell Z, et al. **Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection**. *J Virol* 2009; 83(2):757-769.
50. Doria-Rose NA, Klein RM, Manion MM, O'Dell S, Phogat A, Chakrabarti B, et al. **Frequency and phenotype of human immunodeficiency virus envelope-specific B cells from patients with broadly cross-neutralizing antibodies**. *J Virol* 2009; 83(1):188-199.
51. Richman DD, Little SJ, Smith DM, Wrin T, Petropoulos C, Wong JK. **HIV evolution and escape**. *Trans Am Clin Climatol Assoc* 2004; 115:289-303.
52. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, et al. **Antibody neutralization and escape by HIV-1**. *Nature* 2003; 422(6929):307-312.
53. Stamatatos L, Morris L, Burton DR, Mascola JR. **Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine?** *Nat Med* 2009; 15(8):866-870.
54. Sanchez-Merino V, Fabra-Garcia A, Gonzalez N, Nicolas D, Merino-Mansilla A, Manzardo C, et al. **Detection of Broadly Neutralizing Activity Within the First Months of HIV-1 Infection**. *J Virol* 2016.
55. Moldt B, Rakasz EG, Schultz N, Chan-Hui PY, Swiderek K, Weisgrau KL, et al. **Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo**. *Proc Natl Acad Sci U S A* 2012; 109(46):18921-18925.
56. Pietzsch J, Gruell H, Bournazos S, Donovan BM, Klein F, Diskin R, et al. **A mouse model for HIV-1 entry**. *Proc Natl Acad Sci U S A* 2012; 109(39):15859-15864.
57. Pegu A, Yang ZY, Boyington JC, Wu L, Ko SY, Schmidt SD, et al. **Neutralizing antibodies to HIV-1 envelope protect more effectively in vivo than those to the CD4 receptor**. *Sci Transl Med* 2014; 6(243):243ra288.
58. Ledgerwood JE, Coates EE, Yamshchikov G, Saunders JG, Holman L, Enama ME, et al. **Safety, pharmacokinetics and neutralization of the broadly neutralizing HIV-1 human monoclonal antibody VRC01 in healthy adults**. *Clin Exp Immunol* 2015; 182(3):289-301.
59. Siliciano JD, Siliciano RF. **Latency and viral persistence in HIV-1 infection**. *J Clin Invest* 2000; 106(7):823-825.
60. Munier CM, Kelleher AD, Kent SJ, De Rose R. **The role of T cell immunity in HIV-1 infection**. *Curr Opin Virol* 2013; 3(4):438-446.
61. Ortiz AM, Klatt NR, Li B, Yi Y, Tabb B, Hao XP, et al. **Depletion of CD4(+) T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques**. *J Clin Invest* 2011; 121(11):4433-4445.
62. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, et al. **Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy**. *Nat Med* 1999; 5(5):512-517.
63. International ASSWGoHIVC, Deeks SG, Autran B, Berkhout B, Benkirane M, Cairns S, et al. **Towards an HIV cure: a global scientific strategy**. In: *Nat Rev Immunol*. England; 2012. pp. 607-614.
64. Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, et al. **Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia**. *Science* 1997; 278(5342):1447-1450.
65. Rosenberg ES, Altfeld M, Poon SH, Phillips MN, Wilkes BM, Eldridge RL, et al. **Immune control of HIV-1 after early treatment of acute infection**. *Nature* 2000; 407(6803):523-526.
66. Soghoian DZ, Jessen H, Flanders M, Sierra-Davidson K, Cutler S, Pertel T, et al. **HIV-specific cytolytic CD4 T cell responses during acute HIV infection predict disease outcome**. *Sci Transl Med* 2012; 4(123):123ra125.
67. Demers KR, Reuter MA, Betts MR. **CD8(+) T-cell effector function and transcriptional regulation during HIV pathogenesis**. *Immunol Rev* 2013; 254(1):190-206.
68. Kull FC, Jr. **The TNF receptor in TNF-mediated cytotoxicity**. *Nat Immun Cell Growth Regul* 1988; 7(5-6):254-265.

69. Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, et al. **Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes**. *J Exp Med* 1991; 173(5):1099-1109.
70. Bots M, Medema JP. **Granzymes at a glance**. *J Cell Sci* 2006; 119(Pt 24):5011-5014.
71. Voskoboinik I, Smyth MJ, Trapani JA. **Perforin-mediated target-cell death and immune homeostasis**. *Nat Rev Immunol* 2006; 6(12):940-952.
72. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. **Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection**. *J Virol* 1994; 68(9):6103-6110.
73. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV, et al. **The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection**. *J Exp Med* 2009; 206(6):1253-1272.
74. Jones RB, Walker BD. **HIV-specific CD8(+) T cells and HIV eradication**. *J Clin Invest* 2016; 126(2):455-463.
75. Deng K, Pertea M, Rongvaux A, Wang L, Durand CM, Ghiaur G, et al. **Broad CTL response is required to clear latent HIV-1 due to dominance of escape mutations**. *Nature* 2015; 517(7534):381-385.
76. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, et al. **Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques**. *J Exp Med* 1999; 189(6):991-998.
77. Buchbinder SP, Katz MH, Hessol NA, O'Malley PM, Holmberg SD. **Long-term HIV-1 infection without immunologic progression**. *Aids* 1994; 8(8):1123-1128.
78. Deeks SG, Walker BD. **Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy**. *Immunity* 2007; 27(3):406-416.
79. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. **HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells**. *Blood* 2006; 107(12):4781-4789.
80. Hersperger AR, Pereyra F, Nason M, Demers K, Sheth P, Shin LY, et al. **Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control**. *PLoS Pathog* 2010; 6(5):e1000917.
81. Hersperger AR, Martin JN, Shin LY, Sheth PM, Kovacs CM, Cosma GL, et al. **Increased HIV-specific CD8+ T-cell cytotoxic potential in HIV elite controllers is associated with T-bet expression**. *Blood* 2011; 117(14):3799-3808.
82. Kahan SM, Wherry EJ, Zajac AJ. **T cell exhaustion during persistent viral infections**. *Virology* 2015; 479-480:180-193.
83. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. **PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression**. *Nature* 2006; 443(7109):350-354.
84. Lodi S, Phillips A, Logan R, Olson A, Costagliola D, Abgrall S, et al. **Comparative effectiveness of immediate antiretroviral therapy versus CD4-based initiation in HIV-positive individuals in high-income countries: observational cohort study**. *Lancet HIV* 2015; 2(8):e335-343.
85. Garriga C, Garcia de Olalla P, Miro JM, Ocana I, Knobel H, Barbera MJ, et al. **Mortality, Causes of Death and Associated Factors Relate to a Large HIV Population-Based Cohort**. *PLoS One* 2015; 10(12):e0145701.
86. Palella FJ, Jr., Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, et al. **Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators**. *N Engl J Med* 1998; 338(13):853-860.
87. Cain LE, Logan R, Robins JM, Sterne JA, Sabin C, Bansi L, et al. **When to initiate combined antiretroviral therapy to reduce mortality and AIDS-defining illness in HIV-infected persons in developed countries: an observational study**. *Ann Intern Med* 2011; 154(8):509-515.
88. Lundgren JD, Babiker AG, Gordin F, Emery S, Grund B, Sharma S, et al. **Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection**. *N Engl J Med* 2015; 373(9):795-807.

89. **WHO Guidelines Approved by the Guidelines Review Committee**. In: *Guideline on When to Start Antiretroviral Therapy and on Pre-Exposure Prophylaxis for HIV*. Geneva: World Health Organization Copyright (c) World Health Organization 2015.; 2015.

90. Ryom L, Boesecke C, Gisler V, Manzardo C, Rockstroh JK, Puoti M, et al. **Essentials from the 2015 European AIDS Clinical Society (EACS) guidelines for the treatment of adult HIV-positive persons**. *HIV Med* 2016; 17(2):83-88.
91. Churchill D, Waters L, Ahmed N, Angus B, Boffito M, Bower M, et al. **British HIV Association guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy 2015**. *HIV Med* 2016; 17 Suppl 4:s2-s104.
92. Barouch DH, Michael NL. **Accelerating HIV-1 Vaccine Efficacy Trials**. *Cell* 2014; 159(5):969-972.
93. Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF. **Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection**. *J Infect Dis* 2005; 191(5):654-665.
94. Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, et al. **Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand**. *J Infect Dis* 2006; 194(12):1661-1671.
95. Russell ND, Graham BS, Keefer MC, McElrath MJ, Self SG, Weinhold KJ, et al. **Phase 2 study of an HIV-1 canarypox vaccine (vCP1452) alone and in combination with rgp120: negative results fail to trigger a phase 3 correlates trial**. *J Acquir Immune Defic Syndr* 2007; 44(2):203-212.
96. Russell ND, Hudgens MG, Ha R, Havenar-Daughton C, McElrath MJ. **Moving to human immunodeficiency virus type 1 vaccine efficacy trials: defining T cell responses as potential correlates of immunity**. *J Infect Dis* 2003; 187(2):226-242.
97. Nitayaphan S, Pitisuttithum P, Karnasuta C, Eamsila C, de Souza M, Morgan P, et al. **Safety and immunogenicity of an HIV subtype B and E prime-boost vaccine combination in HIV-negative Thai adults**. *J Infect Dis* 2004; 190(4):702-706.
98. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. **Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial**. *Lancet* 2008; 372(9653):1881-1893.
99. Priddy FH, Brown D, Kublin J, Monahan K, Wright DP, Lalezari J, et al. **Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults**. *Clin Infect Dis* 2008; 46(11):1769-1781.
100. Gray GE, Allen M, Moodie Z, Churchyard G, Bekker LG, Nchabeleng M, et al. **Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study**. *Lancet Infect Dis* 2011; 11(7):507-515.
101. McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, Janes H, et al. **HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis**. *Lancet* 2008; 372(9653):1894-1905.
102. Gray G, Buchbinder S, Duerr A. **Overview of STEP and Phambili trial results: two phase IIb test-of-concept studies investigating the efficacy of MRK adenovirus type 5 gag/pol/nef subtype B HIV vaccine**. *Curr Opin HIV AIDS* 2010; 5(5):357-361.
103. Duerr A, Wasserheit JN, Corey L. **HIV vaccines: new frontiers in vaccine development**. *Clin Infect Dis* 2006; 43(4):500-511.
104. Samri A, Bacchus-Souffan C, Hocqueloux L, Avettand-Fenoel V, Descours B, Theodorou I, et al. **Polyfunctional HIV-specific T cells in Post-Treatment Controllers**. *Aids* 2016; 30(15):2299-2302.
105. Moodie Z, Metch B, Bekker LG, Churchyard G, Nchabeleng M, Mlisana K, et al. **Continued Follow-Up of Phambili Phase 2b Randomized HIV-1 Vaccine Trial Participants Supports Increased HIV-1 Acquisition among Vaccinated Men**. *PLoS One* 2015; 10(9):e0137666.
106. Hammer SM, Sobieszczyk ME, Janes H, Karuna ST, Mulligan MJ, Grove D, et al. **Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine**. *N Engl J Med* 2013; 369(22):2083-2092.

107. Anglemyer A, Horvath T, Rutherford G. **Antiretroviral therapy for prevention of HIV transmission in HIV-discordant couples**. *Jama* 2013; 310(15):1619-1620.
108. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, et al. **Antiretroviral Therapy for the Prevention of HIV-1 Transmission**. *N Engl J Med* 2016; 375(9):830-839.
109. Grinsztejn B, Hosseinipour MC, Ribaudo HJ, Swindells S, Eron J, Chen YQ, et al. **Effects of early versus delayed initiation of antiretroviral treatment on clinical outcomes of HIV-1 infection: results from the phase 3 HPTN 052 randomised controlled trial**. *Lancet Infect Dis* 2014; 14(4):281-290.
110. Rodger AJ, Cambiano V, Bruun T, Vernazza P, Collins S, van Lunzen J, et al. **Sexual Activity Without Condoms and Risk of HIV Transmission in Serodifferent Couples When the HIV-Positive Partner Is Using Suppressive Antiretroviral Therapy**. *Jama* 2016; 316(2):171-181.
111. Spira AI, Marx PA, Patterson BK, Mahoney J, Koup RA, Wolinsky SM, et al. **Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques**. *J Exp Med* 1996; 183(1):215-225.
112. Bourry O, Mannioui A, Sellier P, Roucairol C, Durand-Gasselin L, Dereuddre-Bosquet N, et al. **Effect of a short-term HAART on SIV load in macaque tissues is dependent on time of initiation and antiviral diffusion**. *Retrovirology* 2010; 7:78.
113. Otten RA, Smith DK, Adams DR, Pullium JK, Jackson E, Kim CN, et al. **Efficacy of postexposure prophylaxis after intravaginal exposure of pig-tailed macaques to a human-derived retrovirus (human immunodeficiency virus type 2)**. *J Virol* 2000; 74(20):9771-9775.
114. Cardo DM, Culver DH, Ciesielski CA, Srivastava PU, Marcus R, Abiteboul D, et al. **A case-control study of HIV seroconversion in health care workers after percutaneous exposure. Centers for Disease Control and Prevention Needlestick Surveillance Group**. *N Engl J Med* 1997; 337(21):1485-1490.
115. Sperling RS, Shapiro DE, Coombs RW, Todd JA, Herman SA, McSherry GD, et al. **Maternal viral load, zidovudine treatment, and the risk of transmission of human immunodeficiency virus type 1 from mother to infant. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group**. *N Engl J Med* 1996; 335(22):1621-1629.
116. Cresswell F, Waters L, Briggs E, Fox J, Harbottle J, Hawkins D, et al. **UK guideline for the use of HIV Post-Exposure Prophylaxis Following Sexual Exposure, 2015**. *Int J STD AIDS* 2016; 27(9):713-738.
117. McCormack S, Dunn DT, Desai M, Dolling DI, Gafos M, Gilson R, et al. **Pre-exposure prophylaxis to prevent the acquisition of HIV-1 infection (PROUD): effectiveness results from the pilot phase of a pragmatic open-label randomised trial**. *Lancet* 2016; 387(10013):53-60.
118. Molina JM, Capitant C, Spire B, Pialoux G, Cotte L, Charreau I, et al. **On-Demand Preexposure Prophylaxis in Men at High Risk for HIV-1 Infection**. *N Engl J Med* 2015; 373(23):2237-2246.
119. Auvert B, Taljaard D, Lagarde E, Sobngwi-Tambekou J, Sitta R, Puren A. **Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial**. *PLoS Med* 2005; 2(11):e298.
120. Bailey RC, Moses S, Parker CB, Agot K, Maclean I, Krieger JN, et al. **Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomised controlled trial**. *Lancet* 2007; 369(9562):643-656.
121. Gray RH, Kigozi G, Serwadda D, Makumbi F, Watya S, Nalugoda F, et al. **Male circumcision for HIV prevention in men in Rakai, Uganda: a randomised trial**. *Lancet* 2007; 369(9562):657-666.
122. Gray R, Kigozi G, Kong X, Ssempiija V, Makumbi F, Wattya S, et al. **The effectiveness of male circumcision for HIV prevention and effects on risk behaviors in a posttrial follow-up study**. *Aids* 2012; 26(5):609-615.
123. Stover J, Bollinger L, Izazola JA, Loures L, DeLay P, Ghys PD. **What Is Required to End the AIDS Epidemic as a Public Health Threat by 2030? The Cost and Impact of the Fast-Track Approach**. *PLoS One* 2016; 11(5):e0154893.

124. Kripke K, Reed J, Hankins C, Smiley G, Laube C, Njeuhmeli E. **Impact and Cost of Scaling Up Voluntary Medical Male Circumcision for HIV Prevention in the Context of the New 90-90-90 HIV Treatment Targets**. *PLoS One* 2016; 11(10):e0155734.
125. UNAIDS. **90-90-90. An ambitious treatment target to help end the AIDS epidemic.** In: Joint United Nations Programme on HIV/AIDS (UNAIDS); 2014.
126. Sigal A, Kim JT, Balazs AB, Dekel E, Mayo A, Milo R, et al. **Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy**. *Nature* 2011; 477(7362):95-98.
127. Ensoli B, Cafaro A, Monini P, Marcotullio S, Ensoli F. **Challenges in HIV Vaccine Research for Treatment and Prevention**. *Front Immunol* 2014; 5:417.
128. Mylvaganam GH, Silvestri G, Amara RR. **HIV therapeutic vaccines: moving towards a functional cure**. *Curr Opin Immunol* 2015; 35:1-8.
129. Kutzler MA, Jacobson JM. **Treatment interruption as a tool to measure changes in immunologic response to HIV-1**. *Curr Opin HIV AIDS* 2008; 3(2):131-135.
130. Lederman MM, Penn-Nicholson A, Stone SF, Sieg SF, Rodriguez B. **Monitoring clinical trials of therapeutic vaccines in HIV infection: role of treatment interruption**. *Curr Opin HIV AIDS* 2007; 2(1):56-61.
131. Routy JP, Boulassel MR, Nicolette CA, Jacobson JM. **Assessing risk of a short-term antiretroviral therapy discontinuation as a read-out of viral control in immune-based therapy**. *J Med Virol* 2012; 84(6):885-889.
132. Colven R, Harrington RD, Spach DH, Cohen CJ, Hooton TM. **Retroviral rebound syndrome after cessation of suppressive antiretroviral therapy in three patients with chronic HIV infection**. *Ann Intern Med* 2000; 133(6):430-434.
133. Kilby JM, Goepfert PA, Miller AP, Gnann JW, Jr., Sillers M, Saag MS, et al. **Recurrence of the acute HIV syndrome after interruption of antiretroviral therapy in a patient with chronic HIV infection: A case report**. *Ann Intern Med* 2000; 133(6):435-438.
134. Pollard RB, Rockstroh JK, Pantaleo G, Asmuth DM, Peters B, Lazzarin A, et al. **Safety and efficacy of the peptide-based therapeutic vaccine for HIV-1, Vacc-4x: a phase 2 randomised, double-blind, placebo-controlled trial**. *Lancet Infect Dis* 2014; 14(4):291-300.
135. Vardas E, Stanescu I, Leinonen M, Ellefsen K, Pantaleo G, Valtavaara M, et al. **Indicators of therapeutic effect in FIT-06, a Phase II trial of a DNA vaccine, GTU((R))-Multi-HIVB, in untreated HIV-1 infected subjects**. *Vaccine* 2012; 30(27):4046-4054.
136. Pantaleo G, Levy Y. **Therapeutic vaccines and immunological intervention in HIV infection: a paradigm change**. *Curr Opin HIV AIDS* 2016; 11(6):576-584.
137. Banchereau J, Steinman RM. **Dendritic cells and the control of immunity**. *Nature* 1998; 392(6673):245-252.
138. Lapenta C, Santini SM, Logozzi M, Spada M, Andreotti M, Di Pucchio T, et al. **Potent immune response against HIV-1 and protection from virus challenge in hu-PBL-SCID mice immunized with inactivated virus-pulsed dendritic cells generated in the presence of IFN-alpha**. *J Exp Med* 2003; 198(2):361-367.
139. Garcia F, Routy JP. **Challenges in dendritic cells-based therapeutic vaccination in HIV-1 infection Workshop in dendritic cell-based vaccine clinical trials in HIV-1**. In: *Vaccine*. Netherlands: Copyright (c) 2011 Elsevier Ltd. All rights reserved.; 2011. pp. 6454-6463.
140. Garcia F, Climent N, Guardo AC, Gil C, Leon A, Autran B, et al. **A dendritic cell-based vaccine elicits T cell responses associated with control of HIV-1 replication**. *Sci Transl Med* 2013; 5(166):166ra162.
141. Weinberger LS, Burnett JC, Toettcher JE, Arkin AP, Schaffer DV. **Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity**. *Cell* 2005; 122(2):169-182.
142. Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, et al. **Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation**. *J Virol* 1993; 67(1):277-287.

143. Ensoli B, Bellino S, Tripiciano A, Longo O, Francavilla V, Marcotullio S, et al. **Therapeutic immunization with HIV-1 Tat reduces immune activation and loss of regulatory T-cells and improves immune function in subjects on HAART**. *PLoS One* 2010; 5(11):e13540.
144. Bellino S, Tripiciano A, Picconi O, Francavilla V, Longo O, Sgadari C, et al. **The presence of anti-Tat antibodies in HIV-infected individuals is associated with containment of CD4+ T-cell decay and viral load, and with delay of disease progression: results of a 3-year cohort study**. *Retrovirology* 2014; 11:49.
145. Ensoli F, Cafaro A, Casabianca A, Tripiciano A, Bellino S, Longo O, et al. **HIV-1 Tat immunization restores immune homeostasis and attacks the HAART-resistant blood HIV DNA: results of a randomized phase II exploratory clinical trial**. *Retrovirology* 2015; 12:33.
146. Nimmerjahn F, Ravetch JV. **Antibody-mediated modulation of immune responses**. *Immunol Rev* 2010; 236:265-275.
147. Horwitz JA, Halper-Stromberg A, Mouquet H, Gitlin AD, Tretiakova A, Eisenreich TR, et al. **HIV-1 suppression and durable control by combining single broadly neutralizing antibodies and antiretroviral drugs in humanized mice**. *Proc Natl Acad Sci U S A* 2013; 110(41):16538-16543.
148. Caskey M, Klein F, Lorenzi JC, Seaman MS, West AP, Jr., Buckley N, et al. **Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117**. *Nature* 2015; 522(7557):487-491.
149. Zhou T, Zhu J, Wu X, Moquin S, Zhang B, Acharya P, et al. **Multidonor analysis reveals structural elements, genetic determinants, and maturation pathway for HIV-1 neutralization by VRC01-class antibodies**. *Immunity* 2013; 39(2):245-258.
150. Lynch RM, Boritz E, Coates EE, DeZure A, Madden P, Costner P, et al. **Virologic effects of broadly neutralizing antibody VRC01 administration during chronic HIV-1 infection**. *Sci Transl Med* 2015; 7(319):319ra206.
151. Brown TR. **I am the Berlin patient: a personal reflection**. *AIDS Res Hum Retroviruses* 2015; 31(1):2-3.
152. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, et al. **Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection**. *Cell* 1996; 86(3):367-377.
153. Hutter G, Nowak D, Mossner M, Ganepola S, Mussig A, Allers K, et al. **Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation**. *N Engl J Med* 2009; 360(7):692-698.
154. Henrich TJ, Hanhauser E, Marty FM, Sirignano MN, Keating S, Lee TH, et al. **Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: report of 2 cases**. *Ann Intern Med* 2014; 161(5):319-327.
155. Saez-Cirion A, Bacchus C, Hocqueloux L, Avettand-Fenoel V, Girault I, Lecuroux C, et al. **Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study**. *PLoS Pathog* 2013; 9(3):e1003211.
156. Persaud D, Gay H, Ziemniak C, Chen YH, Piatak M, Jr., Chun TW, et al. **Absence of detectable HIV-1 viremia after treatment cessation in an infant**. *N Engl J Med* 2013; 369(19):1828-1835.
157. Giacomet V, Trabattoni D, Zanchetta N, Biasin M, Gismondo M, Clerici M, et al. **No cure of HIV infection in a child despite early treatment and apparent viral clearance**. *Lancet* 2014; 384(9950):1320.
158. Whitney JB, Hill AL, Sanisetty S, Penaloza-MacMaster P, Liu J, Shetty M, et al. **Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys**. *Nature* 2014; 512(7512):74-77.
159. Archin NM, Liberty AL, Kashuba AD, Choudhary SK, Kuruc JD, Crooks AM, et al. **Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy**. *Nature* 2012; 487(7408):482-485.
160. Deeks SG. **HIV: Shock and kill**. In: *Nature*. England; 2012. pp. 439-440.
161. Liu MA. **DNA vaccines: an historical perspective and view to the future**. *Immunol Rev* 2011; 239(1):62-84.

162. Jin X, Morgan C, Yu X, DeRosa S, Tomaras GD, Montefiori DC, et al. **Multiple factors affect immunogenicity of DNA plasmid HIV vaccines in human clinical trials**. *Vaccine* 2015; 33(20):2347-2353.
163. Wolff JA, Dowty ME, Jiao S, Repetto G, Berg RK, Ludtke JJ, et al. **Expression of naked plasmids by cultured myotubes and entry of plasmids into T tubules and caveolae of mammalian skeletal muscle**. *J Cell Sci* 1992; 103 (Pt 4):1249-1259.
164. Tregoning JS, Kinnear E. **Using Plasmids as DNA Vaccines for Infectious Diseases**. *Microbiol Spectr* 2014; 2(6).
165. Beard CW, Mason PW. **Out on the farm with DNA vaccines**. *Nat Biotechnol* 1998; 16(13):1325-1328.
166. Van Damme P, Cramm M, Safary A, Vandepapeliere P, Meheus A. **Heat stability of a recombinant DNA hepatitis B vaccine**. *Vaccine* 1992; 10(6):366-367.
167. Schalk JA, Mooi FR, Berbers GA, van Aerts LA, Ovelgonne H, Kimman TG. **Preclinical and clinical safety studies on DNA vaccines**. *Hum Vaccin* 2006; 2(2):45-53.
168. Saade F, Petrovsky N. **Technologies for enhanced efficacy of DNA vaccines**. *Expert Rev Vaccines* 2012; 11(2):189-209.
169. Lhomme E, Richert L, Moodie Z, Pasin C, Kalams SA, Morgan C, et al. **Early CD4+ T Cell Responses Are Associated with Subsequent CD8+ T Cell Responses to an rAd5-Based Prophylactic Prime-Boost HIV Vaccine Strategy**. *PLoS One* 2016; 11(4):e0152952.
170. Patil SD, Rhodes DG, Burgess DJ. **Anionic liposomal delivery system for DNA transfection**. *Aaps j* 2004; 6(4):e29.
171. Jorritsma SH, Gowans EJ, Grubor-Bauk B, Wijesundara DK. **Delivery methods to increase cellular uptake and immunogenicity of DNA vaccines**. *Vaccine* 2016; 34(46):5488-5494.
172. Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, Roger MG, et al. **Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges**. *Immunity* 2011; 34(2):269-280.
173. Aguiar JC, Hedstrom RC, Rogers WO, Charoenvit Y, Sacci JB, Jr., Lanar DE, et al. **Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device**. *Vaccine* 2001; 20(1-2):275-280.
174. Raviprakash K, Ewing D, Simmons M, Porter KR, Jones TR, Hayes CG, et al. **Needle-free Biojector injection of a dengue virus type 1 DNA vaccine with human immunostimulatory sequences and the GM-CSF gene increases immunogenicity and protection from virus challenge in Aotus monkeys**. *Virology* 2003; 315(2):345-352.
175. Williams J, Fox-Leyva L, Christensen C, Fisher D, Schlicting E, Snowball M, et al. **Hepatitis A vaccine administration: comparison between jet-injector and needle injection**. *Vaccine* 2000; 18(18):1939-1943.
176. Graham BS, Enama ME, Nason MC, Gordon IJ, Peel SA, Ledgerwood JE, et al. **DNA vaccine delivered by a needle-free injection device improves potency of priming for antibody and CD8+ T-cell responses after rAd5 boost in a randomized clinical trial**. *PLoS One* 2013; 8(4):e59340.
177. Pertmer TM, Eisenbraun MD, McCabe D, Prayaga SK, Fuller DH, Haynes JR. **Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA**. *Vaccine* 1995; 13(15):1427-1430.
178. Birchall J, Coulman S, Pearton M, Allender C, Brain K, Anstey A, et al. **Cutaneous DNA delivery and gene expression in ex vivo human skin explants via wet-etch micro-fabricated micro-needles**. *J Drug Target* 2005; 13(7):415-421.
179. Daugimont L, Baron N, Vandermeulen G, Pavselj N, Miklavcic D, Jullien MC, et al. **Hollow microneedle arrays for intradermal drug delivery and DNA electroporation**. *J Membr Biol* 2010; 236(1):117-125.
180. Keane-Myers AM, Bell M, Hannaman D, Albrecht M. **DNA electroporation of multi-agent vaccines conferring protection against select agent challenge: TriGrid delivery system**. *Methods Mol Biol* 2014; 1121:325-336.

181. Rols MP. **Mechanism by which electroporation mediates DNA migration and entry into cells and targeted tissues**. *Methods Mol Biol* 2008; 423:19-33.
182. Vasan S, Hurley A, Schlesinger SJ, Hannaman D, Gardiner DF, Dugin DP, et al. **In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers**. *PLoS One* 2011; 6(5):e19252.
183. Nilsson C, Hejdeman B, Godoy-Ramirez K, Tecleab T, Scarlatti G, Brave A, et al. **HIV-DNA Given with or without Intradermal Electroporation Is Safe and Highly Immunogenic in Healthy Swedish HIV-1 DNA/MVA Vaccinees: A Phase I Randomized Trial**. *PLoS One* 2015; 10(6):e0131748.
184. Jacobson JM, Zheng L, Wilson CC, Tebas P, Matining RM, Egan MA, et al. **The Safety and Immunogenicity of an Interleukin-12-Enhanced Multiantigen DNA Vaccine Delivered by Electroporation for the Treatment of HIV-1 Infection**. *J Acquir Immune Defic Syndr* 2016; 71(2):163-171.
185. Kalams SA, Parker SD, Elizaga M, Metch B, Edupuganti S, Hural J, et al. **Safety and comparative immunogenicity of an HIV-1 DNA vaccine in combination with plasmid interleukin 12 and impact of intramuscular electroporation for delivery**. *J Infect Dis* 2013; 208(5):818-829.
186. Combadiere B, Liard C. **Transcutaneous and intradermal vaccination**. *Hum Vaccin* 2011; 7(8):811-827.
187. Lambert PH, Laurent PE. **Intradermal vaccine delivery: will new delivery systems transform vaccine administration?** *Vaccine* 2008; 26(26):3197-3208.
188. Kenney RT, Frech SA, Muenz LR, Villar CP, Glenn GM. **Dose sparing with intradermal injection of influenza vaccine**. *N Engl J Med* 2004; 351(22):2295-2301.
189. Ledgerwood JE, Hu Z, Gordon IJ, Yamshchikov G, Enama ME, Plummer S, et al. **Influenza virus h5 DNA vaccination is immunogenic by intramuscular and intradermal routes in humans**. *Clin Vaccine Immunol* 2012; 19(11):1792-1797.
190. Peachman KK, Rao M, Alving CR. **Immunization with DNA through the skin**. *Methods* 2003; 31(3):232-242.
191. Combadiere B, Vogt A, Mahe B, Costagliola D, Hadam S, Bonduelle O, et al. **Preferential amplification of CD8 effector-T cells after transcutaneous application of an inactivated influenza vaccine: a randomized phase I trial**. *PLoS One* 2010; 5(5):e10818.
192. Combadiere B, Mahe B. **Particle-based vaccines for transcutaneous vaccination**. *Comp Immunol Microbiol Infect Dis* 2008; 31(2-3):293-315.
193. Vogt A, Hadam S, Deckert I, Schmidt J, Stroux A, Afraz Z, et al. **Hair follicle targeting, penetration enhancement and Langerhans cell activation make cyanoacrylate skin surface stripping a promising delivery technique for transcutaneous immunization with large molecules and particle-based vaccines**. *Exp Dermatol* 2015; 24(1):73-75.
194. Vogt A, Mahe B, Costagliola D, Bonduelle O, Hadam S, Schaefer G, et al. **Transcutaneous anti-influenza vaccination promotes both CD4 and CD8 T cell immune responses in humans**. *J Immunol* 2008; 180(3):1482-1489.
195. Blazevic V, Mannik A, Malm M, Sikut R, Valtavaara M, Toots U, et al. **Induction of human immunodeficiency virus type-1-specific immunity with a novel gene transport unit (GTU)-MultiHIV DNA vaccine**. *AIDS Res Hum Retroviruses* 2006; 22(7):667-677.
196. Ilves I, Kivi S, Ustav M. **Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which Is mediated by the viral E2 protein and its binding sites**. *J Virol* 1999; 73(5):4404-4412.
197. Joseph S, Quinn K, Greenwood A, Cope AV, McKay PF, Hayes PJ, et al. **A Comparative Phase I Study of Combination, Homologous Subtype-C DNA, MVA, and Env gp140 Protein/Adjuvant HIV Vaccines in Two Immunization Regimes**. *Front Immunol* 2017; 8:149.
198. Keefer MC, Gilmour J, Hayes P, Gill D, Kopycinski J, Cheeseman H, et al. **A phase I double blind, placebo-controlled, randomized study of a multigenic HIV-1 adenovirus subtype 35 vector vaccine in healthy uninfected adults**. *PLoS One* 2012; 7(8):e41936.

199. Gill DK, Huang Y, Levine GL, Sambor A, Carter DK, Sato A, et al. **Equivalence of ELISpot assays demonstrated between major HIV network laboratories**. *PLoS One* 2010; 5(12):e14330.
200. Streeck H, Frahm N, Walker BD. **The role of IFN-gamma Elispot assay in HIV vaccine research**. *Nat Protoc* 2009; 4(4):461-469.
201. Spentzou A, Bergin P, Gill D, Cheeseman H, Ashraf A, Kaltsidis H, et al. **Viral inhibition assay: a CD8 T cell neutralization assay for use in clinical trials of HIV-1 vaccine candidates**. *J Infect Dis* 2010; 201(5):720-729.
202. Rodriguez WR, Addo MM, Rathod A, Fitzpatrick CA, Yu XG, Perkins B, et al. **CD8+ T lymphocyte responses target functionally important regions of Protease and Integrase in HIV-1 infected subjects**. *Journal of translational medicine* 2004; 2(1):15.
203. Gulick RM, Lalezari J, Goodrich J, Clumeck N, DeJesus E, Horban A, et al. **Maraviroc for previously treated patients with R5 HIV-1 infection**. *N Engl J Med* 2008; 359(14):1429-1441.
204. Schuitemaker H, Koot M, Kootstra NA, Dercksen MW, de Goede RE, van Steenwijk RP, et al. **Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population**. *J Virol* 1992; 66(3):1354-1360.
205. Beyrer C, Abdool Karim Q. **The changing epidemiology of HIV in 2013**. *Curr Opin HIV AIDS* 2013; 8(4):306-310.
206. Excler JL, Robb ML, Kim JH. **HIV-1 vaccines: challenges and new perspectives**. *Hum Vaccin Immunother* 2014; 10(6):1734-1746.
207. Dhalla S, Poole G. **Barriers of enrolment in HIV vaccine trials: a review of HIV vaccine preparedness studies**. *Vaccine* 2011; 29(35):5850-5859.
208. Dhalla S, Poole G. **Motivators to participation in actual HIV vaccine trials**. *AIDS Behav* 2014; 18(2):263-277.
209. The Lancet O. **#trial: Clinical research in the age of social media**. *Lancet Oncol* 2014; 15(6):539.
210. Allsworth JE. **Invited commentary: recruiting for epidemiologic studies using social media**. *Am J Epidemiol* 2015; 181(10):747-749.
211. Mitchell MS, Goodman JM, Alter DA, Oh PI, Leahey TM, Faulkner GE. **The Feasibility of Financial Incentives to Increase Exercise Among Canadian Cardiac Rehabilitation Patients**. *J Cardiopulm Rehabil Prev* 2016; 36(1):28-32.
212. Tappin D, Bauld L, Purves D, Boyd K, Sinclair L, MacAskill S, et al. **Financial incentives for smoking cessation in pregnancy: randomised controlled trial**. *Bmj* 2015; 350:h134.
213. Cestari TF, Dantas LP, Boza JC. **Acquired hyperpigmentations**. *An Bras Dermatol* 2014; 89(1):11-25.
214. Taylor S, Grimes P, Lim J, Im S, Lui H. **Postinflammatory hyperpigmentation**. *J Cutan Med Surg* 2009; 13(4):183-191.
215. Vachiramon V, Thadanipon K. **Postinflammatory hypopigmentation**. *Clin Exp Dermatol* 2011; 36(7):708-714.
216. Hannaman D, Dupuy LC, Ellefsen B, Schmaljohn CS. **A Phase 1 clinical trial of a DNA vaccine for Venezuelan equine encephalitis delivered by intramuscular or intradermal electroporation**. *Vaccine* 2016; 34(31):3607-3612.
217. Hayes P, Fernandez N, Omosa-Manyonyi G, Mpendo J, Karita E, Ruzagira E, et al. **Assessment of Viral Inhibition Activity in Low Seroprevalent Adenovirus-35 Vectored HIV Vaccines+/- Adjuvanted Protein or Electroporated DNA**. *AIDS Res Hum Retroviruses* 2014; 30 Suppl 1:A188.
218. Hill PC, Brookes RH, Fox A, Jackson-Sillah D, Jeffries DJ, Lugos MD, et al. **Longitudinal assessment of an ELISPOT test for Mycobacterium tuberculosis infection**. *PLoS Med* 2007; 4(6):e192.
219. Kester KE, Cummings JF, Ockenhouse CF, Nielsen R, Hall BT, Gordon DM, et al. **Phase 2a trial of 0, 1, and 3 month and 0, 7, and 28 day immunization schedules of malaria vaccine RTS,S/AS02 in malaria-naive adults at the Walter Reed Army Institute of Research**. *Vaccine* 2008; 26(18):2191-2202.

220. Smith SG, Smits K, Joosten SA, van Meijgaarden KE, Satti I, Fletcher HA, et al. **Intracellular Cytokine Staining and Flow Cytometry: Considerations for Application in Clinical Trials of Novel Tuberculosis Vaccines**. *PLoS One* 2015; 10(9):e0138042.
221. Horton H, Thomas EP, Stucky JA, Frank I, Moodie Z, Huang Y, et al. **Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination**. *J Immunol Methods* 2007; 323(1):39-54.
222. Valentine LE, Piaskowski SM, Rakasz EG, Henry NL, Wilson NA, Watkins DI. **Recognition of escape variants in ELISPOT does not always predict CD8+ T-cell recognition of simian immunodeficiency virus-infected cells expressing the same variant sequences**. *J Virol* 2008; 82(1):575-581.
223. Gray CM, Mlotshwa M, Riou C, Mathebula T, de Assis Rosa D, Mashishi T, et al. **Human immunodeficiency virus-specific gamma interferon enzyme-linked immunospot assay responses targeting specific regions of the proteome during primary subtype C infection are poor predictors of the course of viremia and set point**. *J Virol* 2009; 83(1):470-478.
224. Wong JT, Colvin RB. **Selective reduction and proliferation of the CD4+ and CD8+ T cell subsets with bispecific monoclonal antibodies: evidence for inter-T cell-mediated cytolysis**. *Clin Immunol Immunopathol* 1991; 58(2):236-250.
225. Fauce SR, Yang OO, Effros RB. **Autologous CD4/CD8 co-culture assay: a physiologically-relevant composite measure of CD8+ T lymphocyte function in HIV-infected persons**. *J Immunol Methods* 2007; 327(1-2):75-81.
226. van Drunen Littel-van den Hurk S, Hannaman D. **Electroporation for DNA immunization: clinical application**. *Expert Rev Vaccines* 2010; 9(5):503-517.
227. Bhutiani N, Philips P, Scoggins CR, McMasters KM, Potts MH, Martin RC. **Evaluation of tolerability and efficacy of irreversible electroporation (IRE) in treatment of Child-Pugh B (7/8) hepatocellular carcinoma (HCC)**. *HPB (Oxford)* 2016; 18(7):593-599.
228. Diehl SJ, Rathmann N, Kostrzewa M, Ritter M, Smakic A, Schoenberg SO, et al. **Irreversible Electroporation for Surgical Renal Masses in Solitary Kidneys: Short-Term Interventional and Functional Outcome**. *J Vasc Interv Radiol* 2016.
229. Egemen A, Aksit S, Kurugol Z, Erensoy S, Bilgic A, Akilli M. **Low-dose intradermal versus intramuscular administration of recombinant hepatitis B vaccine: a comparison of immunogenicity in infants and preschool children**. *Vaccine* 1998; 16(16):1511-1515.
230. Mpendo J, Mutua G, Nyombayire J, Ingabire R, Nanvubya A, Anzala O, et al. **A Phase I Double Blind, Placebo-Controlled, Randomized Study of the Safety and Immunogenicity of Electroporated HIV DNA with or without Interleukin 12 in Prime-Boost Combinations with an Ad35 HIV Vaccine in Healthy HIV-Seronegative African Adults**. *PLoS One* 2015; 10(8):e0134287.
231. Yang OO. **Will we be able to 'spot' an effective HIV-1 vaccine?** *Trends Immunol* 2003; 24(2):67-72.
232. Kis EE, Winter G, Myschik J. **Devices for intradermal vaccination**. *Vaccine* 2012; 30(3):523-538.
233. Samji H, Cescon A, Hogg RS, Modur SP, Althoff KN, Buchacz K, et al. **Closing the gap: increases in life expectancy among treated HIV-positive individuals in the United States and Canada**. *PLoS One* 2013; 8(12):e81355.
234. Li JZ, Etemad B, Ahmed H, Aga E, Bosch RJ, Mellors JW, et al. **The size of the expressed HIV reservoir predicts timing of viral rebound after treatment interruption**. *Aids* 2016; 30(3):343-353.
235. Vieillard V, Gharakhanian S, Lucar O, Katlama C, Launay O, Autran B, et al. **Perspectives for immunotherapy: which applications might achieve an HIV functional cure?** In: *Oncotarget*. United States; 2016. pp. 38946-38958.
236. Curno MJ, Rossi S, Hodges-Mameletzis I, Johnston R, Price MA, Heidari S. **A Systematic Review of the Inclusion (or Exclusion) of Women in HIV Research: From Clinical Studies of Antiretrovirals and Vaccines to Cure Strategies**. *J Acquir Immune Defic Syndr* 2016; 71(2):181-188.

237. Thornhill J, Inshaw J, Oomeer S, Kaleebu P, Cooper D, Ramjee G, et al. **Enhanced normalisation of CD4/CD8 ratio with early antiretroviral therapy in primary HIV infection**. *J Int AIDS Soc* 2014; 17(4 Suppl 3):19480.
238. Daskalopoulou M, Rodger A, Phillips AN, Sherr L, Speakman A, Collins S, et al. **Recreational drug use, polydrug use, and sexual behaviour in HIV-diagnosed men who have sex with men in the UK: results from the cross-sectional ASTRA study**. *Lancet HIV* 2014; 1(1):e22-31.
239. Gaydos J, McNally A, Guo R, Vandivier RW, Simonian PL, Burnham EL. **Alcohol abuse and smoking alter inflammatory mediator production by pulmonary and systemic immune cells**. *Am J Physiol Lung Cell Mol Physiol* 2016; 310(6):L507-518.
240. Hegazi A, Lee MJ, Whittaker W, Green S, Simms R, Cutts R, et al. **Chemsex and the city: sexualised substance use in gay bisexual and other men who have sex with men attending sexual health clinics**. *Int J STD AIDS* 2016.
241. Wilkins E, Nelson M, Agarwal K, Awoyemi D, Barnes E, Bhagani S, et al. **British HIV Association guidelines for the management of hepatitis viruses in adults infected with HIV 2013**. *HIV Med* 2013; 14 Suppl 4:1-71.
242. Martin MP, Carrington M. **Immunogenetics of HIV disease**. *Immunol Rev* 2013; 254(1):245-264.
243. Koofhethile CK, Ndhlovu ZM, Thobakgale-Tshabalala C, Prado JG, Ismail N, Mncube Z, et al. **CD8+ T Cell Breadth and Ex Vivo Virus Inhibition Capacity Distinguish between Viremic Controllers with and without Protective HLA Class I Alleles**. *J Virol* 2016; 90(15):6818-6831.
244. Yang H, Wu H, Hancock G, Clutton G, Sande N, Xu X, et al. **Antiviral inhibitory capacity of CD8+ T cells predicts the rate of CD4+ T-cell decline in HIV-1 infection**. *J Infect Dis* 2012; 206(4):552-561.
245. Pereira Bittencourt Passaes C, Bruel T, Decalf J, David A, Angin M, Monceaux V, et al. **Ultrasensitive HIV-1 p24 detects single infected cells and differences in reservoir induction by latency-reversal agents**. *J Virol* 2017.
246. Kardani K, Bolhassani A, Shahbazi S. **Prime-boost vaccine strategy against viral infections: Mechanisms and benefits**. *Vaccine* 2016; 34(4):413-423.
247. Goepfert PA, Elizaga ML, Sato A, Qin L, Cardinali M, Hay CM, et al. **Phase 1 safety and immunogenicity testing of DNA and recombinant modified vaccinia Ankara vaccines expressing HIV-1 virus-like particles**. *J Infect Dis* 2011; 203(5):610-619.
248. Iyer SS, Amara RR. **DNA/MVA Vaccines for HIV/AIDS**. *Vaccines (Basel)* 2014; 2(1):160-178.
249. Joachim A, Nilsson C, Aboud S, Bakari M, Lyamuya EF, Robb ML, et al. **Potent functional antibody responses elicited by HIV-I DNA priming and boosting with heterologous HIV-1 recombinant MVA in healthy Tanzanian adults**. *PLoS One* 2015; 10(4):e0118486.
250. Ulmer JB, Wahren B, Liu MA. **Gene-based vaccines: recent technical and clinical advances**. *Trends Mol Med* 2006; 12(5):216-222.
251. Matloubian M, Concepcion RJ, Ahmed R. **CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection**. *J Virol* 1994; 68(12):8056-8063.
252. Jacobson JM, Pat Bucy R, Spritzler J, Saag MS, Eron JJ, Jr., Coombs RW, et al. **Evidence that intermittent structured treatment interruption, but not immunization with ALVAC-HIV vCP1452, promotes host control of HIV replication: the results of AIDS Clinical Trials Group 5068**. *J Infect Dis* 2006; 194(5):623-632.
253. Abdul-Jawad S, Ondondo B, van Hateren A, Gardner A, Elliott T, Korber B, et al. **Increased Valency of Conserved-mosaic Vaccines Enhances the Breadth and Depth of Epitope Recognition**. *Mol Ther* 2016; 24(2):375-384.
254. Ferguson AL, Mann JK, Omarjee S, Ndung'u T, Walker BD, Chakraborty AK. **Translating HIV sequences into quantitative fitness landscapes predicts viral vulnerabilities for rational immunogen design**. *Immunity* 2013; 38(3):606-617.
255. Ahmed T, Borthwick NJ, Gilmour J, Hayes P, Dorrell L, Hanke T. **Control of HIV-1 replication in vitro by vaccine-induced human CD8(+) T cells through conserved subdominant Pol epitopes**. *Vaccine* 2016; 34(9):1215-1224.

256. Ondondo B, Murakoshi H, Clutton G, Abdul-Jawad S, Wee EG, Gatanaga H, et al. **Novel Conserved-region T-cell Mosaic Vaccine With High Global HIV-1 Coverage Is Recognized by Protective Responses in Untreated Infection**. *Mol Ther* 2016; 24(4):832-842.
257. Mothe B. **Viral control induced by HIVconsv vaccines and romidepsin in early treated individuals.** In. Conference on Retroviruses and Opportunistic Infections (CROI 2017); 2017.
258. Levin Y, Kochba E, Hung I, Kenney R. **Intradermal vaccination using the novel microneedle device MicronJet600: Past, present, and future**. *Hum Vaccin Immunother* 2015; 11(4):991-997.
259. Munseri PJ, Kroidl A, Nilsson C, Joachim A, Geldmacher C, Mann P, et al. **Priming with a simplified intradermal HIV-1 DNA vaccine regimen followed by boosting with recombinant HIV-1 MVA vaccine is safe and immunogenic: a phase IIa randomized clinical trial**. *PLoS One* 2015; 10(4):e0119629.
260. McCoy JR, Mendoza JM, Spik KW, Badger C, Gomez AF, Schmaljohn CS, et al. **A multi-head intradermal electroporation device allows for tailored and increased dose DNA vaccine delivery to the skin**. *Hum Vaccin Immunother* 2014; 10(10):3039-3047.
261. Ita K. **Recent progress in transdermal sonophoresis**. *Pharm Dev Technol* 2015:1-9.
262. Polat BE, Blankschtein D, Langer R. **Low-frequency sonophoresis: application to the transdermal delivery of macromolecules and hydrophilic drugs**. *Expert Opin Drug Deliv* 2010; 7(12):1415-1432.
263. Dahlan A, Alpar HO, Stickings P, Sesardic D, Murdan S. **Transcutaneous immunisation assisted by low-frequency ultrasound**. *Int J Pharm* 2009; 368(1-2):123-128.
264. Ita K. **Transdermal delivery of vaccines - Recent progress and critical issues**. *Biomed Pharmacother* 2016; 83:1080-1088.
265. Djabri A, Guy RH, Delgado-Charro MB. **Transdermal iontophoresis of ranitidine: an opportunity in paediatric drug therapy**. *Int J Pharm* 2012; 435(1):27-32.
266. Ita K. **Transdermal iontophoretic drug delivery: advances and challenges**. *J Drug Target* 2016; 24(5):386-391.
267. Bernardi DS, Bitencourt C, da Silveira DS, da Cruz EL, Pereira-da-Silva MA, Faccioli LH, et al. **Effective transcutaneous immunization using a combination of iontophoresis and nanoparticles**. *Nanomedicine* 2016; 12(8):2439-2448.
268. Roberts MS, Mohammed Y, Pastore MN, Namjoshi S, Yousef S, Alinaghi A, et al. **Topical and cutaneous delivery using nanosystems**. *J Control Release* 2017; 247:86-105.
269. Kohli AK, Alpar HO. **Potential use of nanoparticles for transcutaneous vaccine delivery: effect of particle size and charge**. *Int J Pharm* 2004; 275(1-2):13-17.
270. Castaldello A, Brocca-Cofano E, Voltan R, Triulzi C, Altavilla G, Laus M, et al. **DNA prime and protein boost immunization with innovative polymeric cationic core-shell nanoparticles elicits broad immune responses and strongly enhance cellular responses of HIV-1 tat DNA vaccination**. *Vaccine* 2006; 24(29-30):5655-5669.
271. Cevc G, Blume G. **Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force**. *Biochim Biophys Acta* 1992; 1104(1):226-232.
272. Mishra D, Dubey V, Asthana A, Saraf DK, Jain NK. **Elastic liposomes mediated transcutaneous immunization against Hepatitis B**. *Vaccine* 2006; 24(22):4847-4855.
273. Seya T, Akazawa T, Tsujita T, Matsumoto M. **Role of Toll-like receptors in adjuvant-augmented immune therapies**. *Evid Based Complement Alternat Med* 2006; 3(1):31-38; discussion 133-137.
274. Mkrtichyan M, Ghochikyan A, Movsesyan N, Karapetyan A, Begoyan G, Yu J, et al. **Immunostimulant adjuvant patch enhances humoral and cellular immune responses to DNA immunization**. *DNA Cell Biol* 2008; 27(1):19-24.
275. Li N, Peng LH, Chen X, Nakagawa S, Gao JQ. **Transcutaneous vaccines: novel advances in technology and delivery for overcoming the barriers**. *Vaccine* 2011; 29(37):6179-6190.
276. Fernando GJ, Chen X, Prow TW, Crichton ML, Fairmaid EJ, Roberts MS, et al. **Potent immunity to low doses of influenza vaccine by probabilistic guided micro-targeted skin delivery in a mouse model**. *PLoS One* 2010; 5(4):e10266.

277. Fernando GJ, Chen X, Primiero CA, Yukiko SR, Fairmaid EJ, Corbett HJ, et al. **Nanopatch targeted delivery of both antigen and adjuvant to skin synergistically drives enhanced antibody responses**. *J Control Release* 2012; 159(2):215-221.
278. Kennedy RB, Poland GA. **The top five "game changers" in vaccinology: toward rational and directed vaccine development**. *Omics* 2011; 15(9):533-537.
279. Karahalil B. **Overview of Systems Biology and Omics Technologies**. *Curr Med Chem* 2016; 23(37):4221-4230.
280. Ulmer JB, Mason PW, Geall A, Mandl CW. **RNA-based vaccines**. *Vaccine* 2012; 30(30):4414-4418.
281. Geall AJ, Ulmer JB. **Introduction to RNA-based vaccines and therapeutics**. *Expert Rev Vaccines* 2015; 14(2):151-152.
282. Weide B, Carralot JP, Reese A, Scheel B, Eigentler TK, Hoerr I, et al. **Results of the first phase I/II clinical vaccination trial with direct injection of mRNA**. *J Immunother* 2008; 31(2):180-188.
283. Harmon TM, Fisher KA, McGlynn MG, Stover J, Warren MJ, Teng Y, et al. **Exploring the Potential Health Impact and Cost-Effectiveness of AIDS Vaccine within a Comprehensive HIV/AIDS Response in Low- and Middle-Income Countries**. *PLoS One* 2016; 11(1):e0146387.
284. Moodley N, Gray G, Bertram M. **Projected economic evaluation of the national implementation of a hypothetical HIV vaccination program among adolescents in South Africa, 2012**. *BMC Public Health* 2016; 16:330.
285. Iwuji CC, McGrath N, de Oliveira T, Porter K, Pillay D, Fisher M, et al. **The Art of HIV Elimination: Past and Present Science**. *J AIDS Clin Res* 2015; 6.
286. El-Sadr WM, Lundgren J, Neaton JD, Gordin F, Abrams D, Arduino RC, et al. **CD4+ count-guided interruption of antiretroviral treatment**. *N Engl J Med* 2006; 355(22):2283-2296.
287. Liszewski MK, Yu JJ, O'Doherty U. **Detecting HIV-1 integration by repetitive-sampling Alu-gag PCR**. *Methods* 2009; 47(4):254-260.

Appendices

Appendix 1: FITBiotech peptide sequences

15-mer overlapping peptides (11 amino acid overlap) were custom synthesized by ProImmune (Oxford, UK).

The following procedure was applied to make the peptide stock solutions and pools of peptides:

1. Peptides were dissolved in original vials supplied by ProImmune by adding 150 µl DMSO (Sigma D1435) into each vial, vortex mixed.
2. The amount containing 1 mg of each peptide was taken out from the original vial and diluted in sterile PBS (PAA Laboratories GmbH, H15-002), the volume was adjusted to 0.5 ml, resulting in peptide stock solutions of 2 mg/ml. Vortex mixed. These peptide stock solutions were kept at -80 ºC since Sept. 2008.
3. The peptide pools were prepared from these 2 mg/ml stock solutions, as described separately for each peptide pool.

Epitope-stretch pool (CTL-pool), consists of 24 peptides

Peptide code	***sequence***
FIT.1-197	ITLWQRPLVTIKIGG
HAN-203	ALIEICTEMEKEGKIS
FIT.1-204	CTEMEKEGKISKIGP
HAN-207	AGLKKKKSVTVLDVG
HAN-208	KKKSVTVLDVGDAYF
FIT.1-209	VTVLDVGDAYFSVPL
FIT.1-210	DVGDAYFSVPLDKDF
FIT.1-211	AYFSVPLDKDFRKYT
FIT.1-212	VPLDKDFRKYTAFTI
FIT.1-213	KDFRKYTAFTIPSIN
FIT.1-218	WKGSPAIFQSSMTKI
FIT.1-223	KQNPDIVIYQYMDDL
FIT.1-224	DIVIYQYMDDLYVGS
FIT.1-228	TVQPIVLPEKDSWTV
FIT.1-233	LVGKLNWASQIYAGI
FIT.1-234	LNWASQIYAGIKVKQ
FIT.1-240	REILKEPVHGVYYDP
FIT.1-244	EKEPIVGAETFYVDG
FIT.1-245	IVGAETFYVDGAANR
HAN-250	AGNLWVTVYYGVPV
FIT.1-251	LWVTVYYGVPVWKEA
FIT.1-252	VYYGVPVWKEATTTL
HAN-258	VERYLRDQQLLGIWGCA
FIT.1-263	INQMLRGPGRAFVTI

Making the pool:

24 peptides, take 150 µl of each stock solution 2 mg/ml in PBS, (24 x 0.15 = 3.6 ml), add 11.4 ml PBS to get 15 ml total volume. Concentration of each individual peptide in the pool is 20 µg/ml.
Aliquoted 0.5 ml
Concentration of DMSO in this peptide pool: 2.3%

Rev-peptide pool, consists of 26 peptides

Peptide code	***sequence***
FIT.1-1	AGRSGDSDEELLKTV
FIT.1-2	GDSDEELLKTVRLIK
FIT.1-3	EELLKTVRLIKFLYQ
FIT.1-4	KTVRLIKFLYQSNPP
HAN-5	LIKFLYQSNPPPSNE
HAN-6	LYQSNPPPSNEGTRQ
HAN-7	SNPPPSNEGTRQARRN
HAN-8	SNEGTRQARRNRRRR
FIT.1-9	TRQARRNRRRRWRER
FIT.1-10	RRNRRRRWRERQRQI
FIT.1-11	RRRWRERQRQIRSIS
HAN-12	RERQRQIRSISERIL
HAN-13	RQIRSISERILSTFL
HAN-14	SISERILSTFLGRPA
HAN-15	RILSTFLGRPAEPVP
FIT.1-16	TYLGRPAEPVPLQLP
FIT.1-17	RPAEPVPLQLPPLER
FIT.1-18	PVPLQLPPLERLTLD
HAN-19	LQLPPLERLTLDCSED
HAN-20	LERLTLDCSEDCGNS
HAN-21	TLDCSEDCGNSGTQG
HAN-22	CSEDCGNSGTQGVGSP
HAN-23	GNSGTQGVGSPQVLV
HAN-24	TQGVGSPQVLVESPA
HAN-25	GSPQVLVESPAVLEP
HAN-26	VLVESPAVLEPGTKE

Making the pool:

Take 150 µl of each peptide stock solution 2 mg/ml in PBS, (26 x 0.15 ml= 3.9 ml), add 11.1 ml PBS to get 15 ml total volume. Concentration of each individual peptide in the pool is 20µg/ml. Aliquoted 0.5 ml

Nef-pool, consists of 46-peptides

Peptide code	***sequence***
HAN-30	TSVGKWSKCSGWPTV
HAN-31	KWSKCSGWPTVRERM
HAN-32	CSGWPTVRERMKQAE
HAN-33	PTVRERMKQAEPEPA
HAN-34	ERMKQAEPEPAADGV
HAN-35	QAEPEPAADGVGAAS
HAN-36	EPAADGVGAASRDLE
HAN-37	DGVGAASRDLEKHGA
HAN-38	AASRDLEKHGAITSS
HAN-39	DLEKHGAITSSNTAT
HAN-40	HGAITSSNTATNNAA
HAN-41	TSSNTATNNAACAWL
HAN-42	TATNNAACAWLEAQE
FIT.1-43	CAWLEAQEEEEVGFP
HAN-44	AWLEAQEEEEVGFPV
HAN-45	AQEEEEVGFPVRPQV
HAN-46	EEVGFPVRPQVPLRP
HAN-47	FPVRPQVPLRPMTYK
HAN-48	PQVPLRPMTYKGALD
HAN-49	LRPMTYKGALDLSHF
HAN-50	TYKGALDLSHFLKEK
HAN-51	ALDLSHFLKEKGGLE
HAN-52	SHFLKEKGGLEGLIY
HAN-53	KEKGGLEGLIYSPKR
HAN-54	GLEGLIYSPKRQEIL
HAN-55	LIYSPKRQEILDLWV
HAN-56	PKRQEILDLWVYHTQ
FIT.1-56	**RQDILDLWVYHTQGYF (HAN-57 failed synthesis)**
HAN-58	LWVYHTQGYFPDWQN
HAN-59	HTQGYFPDWQNYTPG
HAN-60	YFPDWQNYTPGPGVR
HAN-61	WQNYTPGPGVRYPLT
HAN-62	TPGPGVRYPLTFGWC
HAN-63	GVRYPLTFGWCFKLV
HAN-64	PLTFGWCFKLVPVEP
HAN-65	GWCFKLVPVEPDEEE
HAN-66	KLVPVEPDEEENSSL
HAN-67	VEPDEEENSSLLHPA failed synthesis
HAN-68	EEENSSLLHPASLHG failed synthesis
HAN-69	SSLLHPASLHGTEDT
HAN-70	HPASLHGTEDTEREV
HAN-71	LHGTEDTEREVLKWK
HAN-72	EDTEREVLKWKFDSH
FIT.1-74	**EVLVWKFDSRLAFHH (HAN 73 failed synthesis)**
HAN-74	KWKFDSHLAFHHKAR
HAN-75	DSHLAFHHKARELHP
HAN-76	AFHHKARELHPEYYK
HAN-77	KARELHPEYYKDCKL

Making the pool:
Take 150 µl of each peptide stock solution 2 mg/ml in PBS, (46 x 0.15 ml= 6.9 ml), add 8.1 ml PBS to get 15 ml total volume. Concentration of each individual peptide in the pool is 20 µg/ml. Aliquoted 0.5 ml

Tat-pool, consists of 23-peptides

Peptide code	***sequence***
FIT.1-81	EPVDPRLEPWKHPGS
HAN-82	PRLEPWKHPGSQPRT
HAN-83	PWKHPGSQPRTPCTN
HAN-84	PGSQPRTPCTNCYCK
HAN-85	PRTPCTNCYCKKCCL
HAN-86	CTNCYCKKCCLHCQV
HAN-87	YCKKCCLHCQVCFTR
HAN-88	CCLHCQVCFTRKGLG
HAN-89	CQVCFTRKGLGISYG
HAN-90	FTRKGLGISYGRKKR
FIT.1-91	GLGISYGRKKRRQRR
FIT.1-92	SYGRKKRRQRRRAPQ
FIT.1-93	KKRRQRRRAPQDSQT
FIT.1-94	RQRRRAPQDSQTHQVS
HAN-95	APQDSQTHQVSLPKQ
HAN-96	SQTHQVSLPKQPSSQ
HAN-97	HQVSLPKQPSSQQRGD
HAN-98	PKQPSSQQRGDPTGP
HAN-99	SSQQRGDPTGPKKSK
HAN-100	RGDPTGPKKSKKKVE
HAN-101	TGPKKSKKKVERETE
HAN-102	KSKKKVERETEADPF
HAN-103	KVERETEADPFDAAV

Making the pool:
Take 150 µl of each peptide stock solution 2 mg/ml in PBS, (23 x 0.15 ml= 3.45 ml), add 11.55 ml PBS to get 15 ml total volume. Concentration of each individual peptide in the pool is 20µg/ml. Aliquoted 0.5 ml

P17/24-pool, consists of 88-peptides

Peptide code	***sequence***
HAN-106	GARASVLSGGELDKW
HAN-107	SVLSGGELDKWEKIR
HAN-108	GGELDKWEKIRLRPG
HAN-109	DKWEKIRLRPGGKKK
HAN-110	KIRLRPGGKKKYQLK
HAN-111	RPGGKKKYQLKHIVW
HAN-112	KKKYQLKHIVWASRE
HAN-113	QLKHIVWASRELERF
FIT.1-114	IVWASRELERFAVNP
FIT.1-115	SRELERFAVNPGLLE
FIT.1-116	ERFAVNPGLLETSEG
FIT.1-117	VNPGLLETSEGCRQI
HAN-118	LLETSEGCRQIMGQL
HAN-119	SEGCRQIMGQLQPSL
HAN-120	RQIMGQLQPSLQTGS
FIT.1-121	GQLQPSLQTGSEELR
FIT.1-122	PSLQTGSEELRSLYN
FIT.1-123	TGSEELRSLYNTVAT
FIT.1-124	ELRSLYNTVATLYCV
FIT.1-125	LYNTVATLYCVHQKI
FIT.1-126	VATLYCVHQKIEVKD
FIT.1-127	YCVHQKIEVKDTKEA
HAN-128	HQKIEVKDTKEALDKV
HAN-129	VKDTKEALDKVEEEQ
HAN-130	KEALDKVEEEQNNSK
HAN-131	DKVEEEQNNSKKKAQ
HAN-132	EEQNNSKKKAQQEAA
HAN-133	NSKKKAQQEAADAGN
HAN-134	KAQQEAADAGNRNQV
HAN-135	EAADAGNRNQVSQNY
HAN-136	AGNRNQVSQNYPIVQ
FIT.1-137	SQVSQNYPIVQNLQG
FIT.1-138	SQNYPIVQNLQGQMVH
FIT.1-139	IVQNLQGQMVHQAIS
FIT.1-140	LQGQMVHQAISPRTL
FIT.1-141	MVHQAISPRTLNAWV
FIT.1-142	AISPRTLNAWVKVVE
FIT.1-143	RTLNAWVKVVEEKAF
FIT.1-144	AWVKVVEEKAFSPEV
FIT.1-145	VVEEKAFSPEVIPMF
FIT.1-146	KAFSPEVIPMFSALS
FIT.1-147	PEVIPMFSALSEGAT
FIT.1-148	PMFSALSEGATPQDL
FIT.1-149	ALSEGATPQDLNTML
FIT.1-150	GATPQDLNTMLNTVG
FIT.1-151	PQDLNTMLNTVGGHQA
FIT.1-152	TMLNTVGGHQAAMQM
FIT.1-153	TVGGHQAAMQMLKET
FIT.1-154	HQAAMQMLKETINEE

FIT.1-155	MQMLKETINEEAAEW
FIT.1-156	KETINEEAAEWDRLH
FIT.1-157	INEEAAEWDRLHPVHA
FIT.1-158	AEWDRLHPVHAGPIA
FIT.1-159	RLHPVHAGPIAPGQM
FIT.1-160	VHAGPIAPGQMREPR
FIT.1-161	PIAPGQMREPRGSDI
FIT.1-162	GQMREPRGSDIAGTT
FIT.1-163	EPRGSDIAGTTSTLQ
FIT.1-164	SDIAGTTSTLQEQIG
FIT.1-165	GTTSTLQEQIGWMTN
FIT.1-166	TLQEQIGWMTNNPPI
FIT.1-167	EQIGWMTNNPPIPVGE
FIT.1-168	MTNNPPIPVGEIYKR
FIT.1-169	PPIPVGEIYKRWIIL
FIT.1-170	VGEIYKRWIILGLNK
FIT.1-171	YKRWIILGLNKIVRM
FIT.1-172	IILGLNKIVRMYSPT
FIT.1-173	LNKIVRMYSPTSILD
HAN-174	VRMYSPTSILDIKQG
HAN-175	SPTSILDIKQGPKEP
HAN-176	ILDIKQGPKEPFRDY
HAN-177	KQGPKEPFRDYVDRF
FIT.1-178	KEPFRDYVDRFYKTL
FIT.1-179	RDYVDRFYKTLRAEQ
HAN-180	DRFYKTLRAEQATQE
HAN-181	KTLRAEQATQEVKNW
HAN-182	AEQATQEVKNWMTET
HAN-183	TQEVKNWMTETLLVQ
FIT.1-184	KNWMTETLLVQNANP
FIT.1-185	TETLLVQNANPDCKT
FIT.1-186	LVQNANPDCKTILKA
FIT.1-187	ANPDCKTILKALGPA
FIT.1-188	CKTILKALGPAATLE
FIT.1-189	LKALGPAATLEEMMT
FIT.1-190	GPAATLEEMMTACQG
FIT.1-191	TLEEMMTACQGVGGP
FIT.1-192	MMTACQGVGGPGHKA
FIT.1-193	CQGVGGPGHKARVLP

Making the pool:

Take 150 µl of each peptide stock solution 2 mg/ml in PBS, (88 x 0.15 ml= 13.2 ml), add 1.8 ml PBS to get 15 ml total volume.

Concentration of each individual peptide in the pool is 20 µg/ml. Aliquoted 0.5 ml

Concentration of DMSO in the peptide pool: 8.9%

Appendix 2: IFN-ɣ T cell ELISpot SOP, GcLP, Imperial College London

Day 0: Preparation of PBMCs 24 hours prior to ELISpot assay

PBMC vials at a concentration of 1 x 10^7cells were removed from vapour phase nitrogen and thawed in a water bath at 37°C, until a small amount of ice remained. The cryovials containing cells were then removed, sprayed with 70% ethanol, dried and transferred to a sterile class II safety cabinet. Using a sterile pastette, each thawed cell suspension (corresponding to an individual trial participant), was transferred to a pre-labelled 15ml falcon tube containing 9mls of R20 at room temperature. The tubes were then centrifuged at room temperature for 10 minutes at 400*g* with the brake on.

The supernatant was decanted and the tube tapped to loosen the cell pellet. Using a sterile pastette, the cells were then re-suspended with 1ml of R20, topped up to 4mls with R20 and transferred to 2 wells of a pre-labelled 24 well plate. The plates containing the PBMC were rested by incubating overnight at 37°C/5% $C0_2$.

Day 1: ELISpot assay using rested PBMCs for 24 hours

Pre-coated Mabtech 96-well ELISpot plates were removed from their packaging and washed 5 times with 200µl/well of sterile PBS using a multi-channel pipette. Wash steps were performed under aseptic conditions in the class II safety cabinet. 200µl/well of R10 was then added to the plates for blocking at room temperature for at least 1 hour.

After incubation overnight, the cells were re-suspended from the bottom of the wells using a sterile pastette and transferred to a sterile 15ml labelled falcon tube. 5mls of R10 assay medium (at room temperature) was then used to rinse wells and the cell suspension added to the corresponding tube. The tubes were then centrifuged at room temperature for 10 minutes at 400*g* with the brake on.

The supernatant was discarded (taking care not to lose the cell pellet) and the tube tapped to loosen the cell pellet as in Day 0. An additional 1ml of R10 was added to each tube and cells re-suspended thoroughly using a sterile pastette.

A total PBMC and viable PBMC cell count was then conducted and cell viability recorded (Figure 2.1). A worked example is shown below:

Total count = 9.4 x 10^6 PBMC per ml
Viability = 94%
Viable count = 9.4 x 10^6 x 0.94 = 8.8 x 10^6 PBMC/ml
Therefore the total number of live PBMC will be the total volume multiplied by the viable count.
Total PBMC = 8.8 x 10^6 x 1.2ml = 10.6 x 10^6 Total PBMC
The PBMC concentration was then adjusted to 4 x 10^6 PBMC/ml with R10 as shown below for this worked example.
10.6 x 10^6/4 x 10^6 = 2.7ml

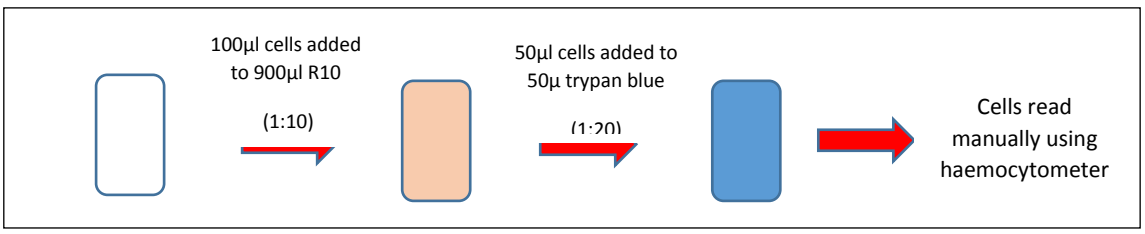


Figure 2.1 Diagram to show cell concentrations used for total and viable cell count.

Before the end of plate blocking, the stimulation media including positive and negative controls was prepared. For the negative control R10 was used plus cells alone with no stimulation. Once the required number of viable PBMCs were obtained and the stimulation media prepared, the R10 blocking solution was removed from the plates. For the PHA positive control wells, cells were further

diluted down to a concentration of 1:10 to enable spot forming cells (SFC) to be read accurately with the plate reader. 50µl/well in triplicate of stimulation media was then added to wells according to the pre-defined plate layout shown below (Figure 2.2), and then 50µl/well in triplicate wells of the correct concentration of the cells were added on top of the stimulation media. The plates were then incubated for 16-24 hours at 37°C/5% $C0_2$.

1	2	3	4	5	6	7	8	9	10	11	12
NEG			NEG			NEG			NEG		
REV			REV			REV			REV		
TAT			TAT			TAT			TAT		
NEF			NEF			NEF			NEF		
GAG			GAG			GAG			GAG		
CTL			CTL			CTL			CTL		
PHA			PHA			PHA			PHA		
CEFT			CEFT			CEFT			CEFT		

Figure 2.2: Plate layout where each row has one sample in triplicate. The negative wells denote media plus cells. PHA and CEFT are both positive controls.

Day 2: Development of ELISpot assay

Just before the end of the 16-24 hour incubation period, mouse-anti human IFN-ɣ detection antibody was prepared in sterile 0.5% FCS/PBS solution, and filtered using a 0.2µm filter. The plates were then removed from the incubator and washed 5 times with 200µl/well of sterile PBS using a multi-channel pipette. The detection antibody prepared above was added to all wells (100µl/well) and plates incubated at room temperature for 2 hours.

After 2 hours the plates were washed a further 5 times with 200µl/well of sterile PBS to remove any unbound primary antibody and 100µl/well of Streptavidin-ALP solution in 0.5% PBS/FCS added to each well of the plates and incubated for a further hour at room temperature.

Following the incubation period, the plates were washed as per previous steps, and 100µl/well of filtered substrate (BCIP/NBT) added. This substrate solution was left to develop until the appearance of distinct spots could be seen (usually between 4-6 minutes, with a maximum of 10 minutes). Colour development was stopped by extensive washing of the plates with cold water.

Developed plates were then left to dry in the dark at room temperature for 24-48 hours and read using an automated AID iSPOT ELISpot plate reader using a pre-defined programme for the detection of IFN-ɣ spot forming cells in human trial samples.

Appendix 3: Intracellular cytokine staining SOP, GcLP, Imperial College London

This standard operational procedure (SOP) describes the procedure for the staining of Peripheral Blood Mononuclear Cells (PBMC) for T cell and intracellular cytokine analysis for vaccine studies conducted by the Immunology Core Laboratory at Imperial College London, St Mary's Campus.

All reagents were prepared as follows:

R10 Media- prepared as described in Methods: Chapter 2

Assay buffer

Assay buffer consists of PBS x 1 with 5% FCS and 0.05% sodium azide

Prepare assay buffer by adding 25ml (5%) FCS and 0.25g sodium azide to 475ml PBS.

PMA/Ionomycin Stimulation Media

To a 1mg/mL (1000µg/mL) solution of PMA take:

- 5µL of 1mg/mL PMA and 245µL of PBS: 20µg/mL
- Take 100µL of 20µg/mL solution and add 400µL of PBS:4µg/mL (4000ng/mL)

To a 1mg/mL (1000µg/mL) solution of ionomycin take:

- 10µL of 1mg/mL ionomycin and 15µL of PBS: 400µg/mL

Peptide pools- prepared as described in Methods: Chapter 2

Brefeldin A Solution

Supplied at 1000x working concentration. Prior to addition to the cell cultures, a 1:1000 dilution in the culture media should be made.

- For a 1x solution: dilute 1 in 10 prior to addition: add 25µL of Brefeldin A to 225µL of culture media.

Near-Infrared eFluor Viability dye

Bring one vial of the fluorescent reactive dye to room temperature before removing the cap.
Can be freeze/thawed up to 20 times when stored at ≤–20°C, protected from light and moisture.

Dilute to 1:20 working concentration with PBS:

- Add 5µL of dye to 95µL of PBS. Add 2µL of working solution to each sample.

Fc Block

2% Fc block preparation:

- Per 5mL of Fc block required: Add 200µL of human serum to 9800µL of PBS

1x Permeabilization Buffer (Perm Buffer)

Supplied as a 10 x concentrate. Precipitates are commonly visible. Buffer can be brought to room temperature and mixed. Allow any precipitates to settle out before making the 1x solution. Prior to use, this should be diluted 10-fold in distilled water. 1x Permeabilization Buffer contains 0.1% saponin and 0.009% sodium azide and can be filtered if precipitates are visible.

- To prepare 10mL of 1 x perm buffer : Add 1mL of 10x perm buffer to 9mL of distilled water.

3% Paraformaldehyde (PFA)

Prepare 3% paraformaldehyde by diluting 10% PFA 3:7 in 1X DPBS by adding 30mL of 10% paraformaldehyde to 70mL DPBS. Stable for 6 months at room temperature.

Procedure

All pipetting and mixing was carried out in a biological class II safety cabinet. Unless otherwise stated the procedure is carried out at room temperature (15-30°C).

Thawing of PBMC samples (day 1)

PBMCs were thawed according to SOP GcLP Lab/SOP. Cells were resuspended at a concentration of 1 x 10^6/mL in R10 media and rested overnight at 37°C in a CO_2 incubator.

Cell stimulation (day 2)

Cells were removed from the incubator, transferred into a Falcon tube and centrifuged at 400*g* for 5 minutes at room temperature with full brakes on. The supernatant was aspirated carefully and discarded.

Resuspended cells in 1mL of R10 media were counted manually and made up to a concentration of 10 x10^6 cells/mL.

Prepare the appropriate stimulation mixes as detailed below:

- 0.025% DMSO in negative control
- PMA at a final concentration of 10ng/mL and ionomycin at a final concentration of 1µg/mL
- 5 pre-diluted peptide pools at a final concentration of 2.5µg/mL per well with 1µg/mL CD28/CD49d.

Condition	Pre-diluted Peptide pool	PMA	Iono	CD28/ CD49d	DMSO	Media (R10)	Total
No stimulus	0	0	0	0	1µL	1999µl	**2000µl**
Positive control	0	15µL	15µL	0	0	2970µl	**3000µl**
Peptide pools	75µl	0	0	15µL	0	1410µl	**1500µl**

Add 100µL of PBMC (at a concentration of 10 x10^6 cells/mL i.e. 1x10^7/ml) and 100µL of the stimulus reagents to the appropriate wells of a 96 well plate/tubes and incubate for 6 hours ± 15 minutes at 37 °C, 5% CO_2.

After 2 hours ± 15 minutes of stimulation, add 2µL/well of the Brefeldin A solution (final concentration of 1x) to the cell culture.

After stimulation; centrifuge the plates/tubes at 400*g* for 5 minutes at room temperature with full brake. Aspirate supernatant carefully. Resuspend the cells in 100µL PBS/well.

Staining Procedure

Add 2µL of the NIR viability dye at working concentration to all samples, except unstained, and mix well. Incubate at room temperature or on ice for 30± 5 minutes, protected from light.

Wash the cells with 100µL/well of assay buffer. Centrifuge at room temperature at 400x*g* for 5 minutes with the brake on. Aspirate supernatant.

Reconstitute cells in 80µL of 2% Fc block and incubate for at least 5 minutes.

Add 20µL of surface staining antibody cocktail (see worksheet for preparation details) for fully stained samples and appropriate FMOs (Fluorescence minus one; tube containing all antibodies except the antibody of interest to establish correct gating during acquisition) and incubate for 20-30 minutes in the dark at 4°C.

Wash cells using 100µL/well of assay buffer and centrifuge at 400 x*g* for 5 minutes. Aspirate supernatant. Resuspend pellet in a total of 100µL assay buffer (prepared as described above).

Fix cells by adding 100µL of IC fixation buffer per well/tube and mix well. Incubate in the dark at room temperature for 20-60 minutes.

Centrifuge plates (or tubes) at room temperature at 400x*g* for 5 minutes with the brake on. Aspirate supernatant.

Add 200µL of 1x Permeabilization Buffer to the cells. Centrifuge samples at 400x*g* at room temperature for 5 minutes, then carefully discard the supernatant.

Resuspend cells in 50µL of 1x Permeabilization Buffer. Add 50µL of intracellular antibody cocktail to fully stained samples and appropriate FMOs and incubate for 20-30 minutes in the dark at room temperature.

Add 100µL 1x Permeabilization Buffer to each well. Centrifuge samples at 400x*g* at room temperature for 5 minutes, then discard the supernatant.

Add 200µL Assay Buffer to each well. Centrifuge samples at 400x*g* at room temperature for 5 minutes, then discard the supernatant.

Resuspend cells in 200 µL of fixation buffer (3% PFA or fixation buffer) and store at 2-8°C for up to 18 hours before analyzing.

Compensation samples

Compensation samples can be run using compensation beads or the cells of interest.

This is the protocol for use with BD compensation particles (Note: these particles only bind κ antibodies):

- To 100µL of assay buffer add 1 drop of negative control beads and one drop of Anti-mouse Ig beads (vortex well before and after adding) for each compensation tube.

- Add the volume used in the sample of each undiluted fluorochrome conjugated antibody to the appropriately labelled tube and incubate for 15-30 minutes at room temperature in the dark.

- Add 2mL of PBS to each tube and centrifuge for 5 minutes at 400x*g*. Aspirate supernatant carefully.

- Resuspend in 0.5 mL of fixation buffer.

Analysis

1. Filter all samples prior to analysis into a 1.4 mL U-bottom tube and place inside a 5mL FACS tube before running. Add additional PBS/assay buffer when filtering to ensure maximum cell recovery in each tube.

2. For initial set-up; create a new experiment and use the highest concentration of each antibody to establish the correct fluorescent voltage before recording events. Save this as a template and as Application Settings for future use.

3. For analysis after initial set-up, create a new experiment from the previous template and apply saved Application settings. Ensure the voltages have updated according to the daily CST values for each experiment.

4. Ensure the appropriate quality control checks have been run prior to acquisition of any data. This includes that the CST check has been performed that day and has passed and run 8 peak beads to ensure all signals from the 7^{th} peak for each fluorescent channel fall within the acceptable gates.

5. Run compensation samples to set the compensation matrix for fully stained samples. Use FMOs to establish correct gates have been set. Label all samples fully in Diva software.

6. Aim to record 50,000 live CD3+ cells for each sample; any samples where this number is not recorded will be annotated on the raw data.

7. Samples are to be analysed on a BD LSRFortessa operating FACS Diva software and the FCS files are to be exported immediately after acquisition of samples.

8. Analyse the data using a flow cytometry analysis program such as FlowJo.

Appendix 4: VIA optimization

This was conducted using the following steps:

A 'mock' VIA run was set up where all steps were conducted as per VIA below until Day 7 (infection day). At day 7, cells were stained and read using flow cytometry. Therefore, the first few steps described below are an adapted version of the full VIA I conducted, but using donor cones instead of trial samples.

PBMC samples from donor cones were thawed as described previously and transferred to 50mls tubes containing 9mls R10 media. The cell were counted manually using a haemocytometer, and centrifuged at 250*g* for 10 minutes. The supernatant was decanted and cells resuspended and adjusted to $1.5x10^6$ PBMC per ml with R10.

To generate a pure population of CD4+ target cells, 6mls of the PBMC suspension from each donor was added to a T75 flask with 60µL of CD3/8+ bispecific antibody (at 50µg/ml, final concentration = 0.5µg/ml), and 30µL IL-2 (10 units/µL, final concentration = 50 units/ml). To generate a pure population of CD8+ effector cells, 3mls of the PBMC suspension from each donor was added to a T25 flask with 30µL of CD3/4+ bispecific antibody (at 50µg/ml, final concentration = 0.5µg/ml), and 15µL IL-2 (10 units/µL, final concentration = 50 units/ml). All flasks were mixed well at this point. The flasks were then incubated upright at 37°C/5% $C0_2$.

On days 3 and 6, the culture volume was doubled using R10/50 (6 or 3mls on day 3, 12 or 6mls on day 6 depending on size of flask) with incubations continued, this time placing the flasks horizontal to allow expansion and mixing of contents.

On day 7 the cells were recovered and transferred into 50ml tubes for staining, centrifuged at 400*g* for 5 minutes and washed and centrifuged again in 5mls of PBS. The cells were then resuspended in 1ml PBS, counted and made up to a concentration of $5x10^6$ cells/mL in PBS ready for staining via the procedure outline below:

1. 2µL viability dye was added to 100µL of the cell suspension in a FACS tube and incubated at room temperature for 30 minutes in the dark
2. After 30 minutes the cells were washed with 500µL assay buffer and centrifuged at 400*g* for 5 minutes following which the supernatant was aspirated
3. Next 20µL of surface staining antibody cocktail was added to the stained samples and incubated for a further 20-30 minutes at room temperature in the dark
4. Step 2 repeated
5. Cells were resuspended in 300µL fixation buffer and stored in the fridge for up to 18 hours before analysing.

For the compensation samples 1 drop of negative control beads and 1 drop of anti-mouse Ig beads were added to 100µL of assay buffer in 2 separate FACS tubes. The same volume used for the staining of undiluted flurochrome conjugated antibody was added to the appropriately labelled tube and incubated at room temperature for 15-30mins in the dark. 2mls PBS was then added to each tube, the cells centrifuged for 5 minutes at 400*g*, supernatant aspirated and cells resuspended in 0.5ml fixation buffer. The cells and compensation tubes were then ready to be read using the flow cytometer.

Optimisation results from the CD4+ and CD8+ cell cultures using flow cytometry

Donor cones were used for optimisation and flow cytometry was conducted to analyse the CD4+ and CD8+ ratios at baseline (day 0), and days 7, 10, and 14 post expansion using IL-2 and the bispecific antibodies. 2 different batches of the bispecific antibodies were used in the optimisation panel shown below neither of which had an effect on generation of pure populations of either CD4+ or CD8+ cells. To further attempt to overcome these challenges I trialed different concentrations of the bispecifics (0.5µg/ml and 1µg/ml), as well as comparing the use of flasks and 12 well plates for expansion in order to see if certain conditions were affecting the experiment. No significant change in the day 14 CD4+ and CD8+ pure populations were seen despite all these optimisation conditions, suggesting these bispecifics were unable to adequately generate and expand pure populations of a >90% target. The graphs below show flow cytometry data using the 2 different batches, including the gating strategy used.

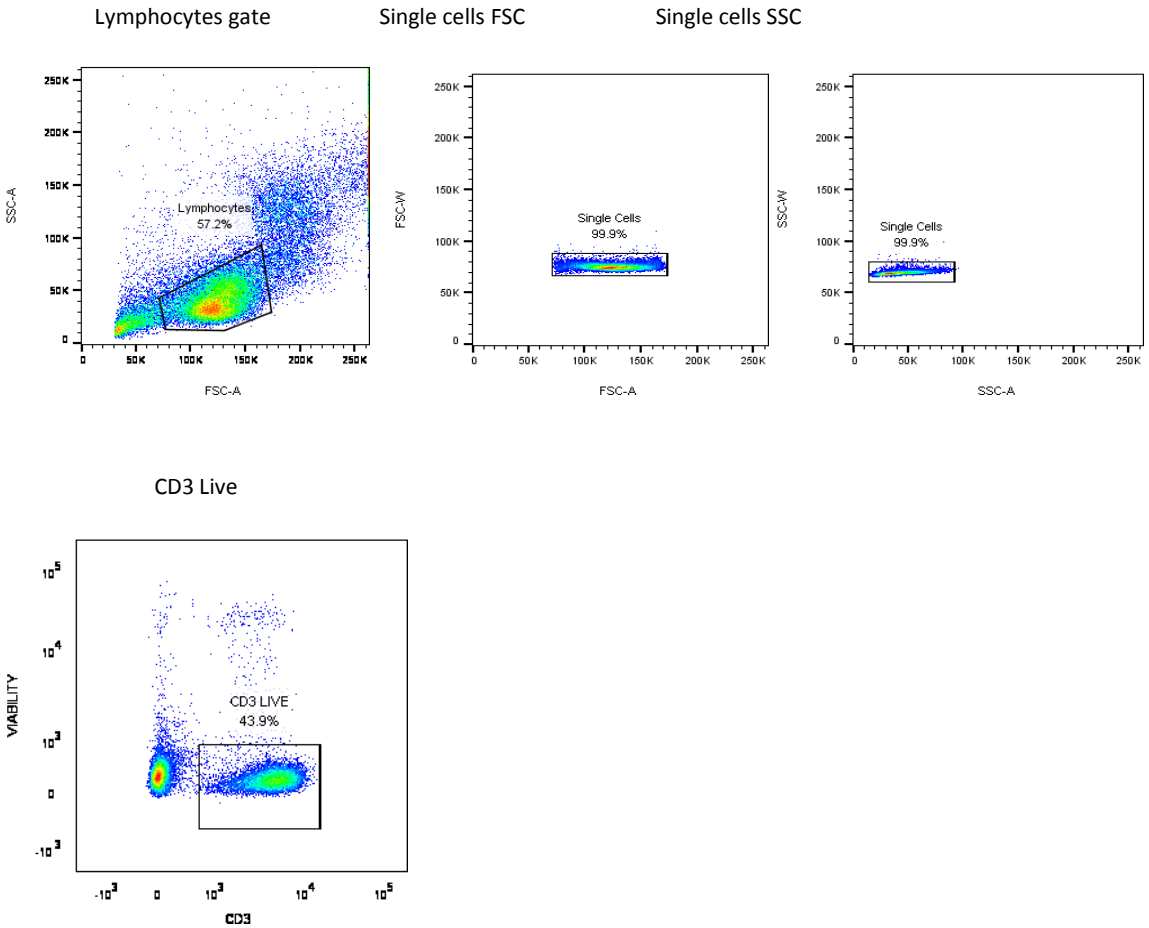


Figure 4.1: Gating strategy use for flow cytometry

Day 0

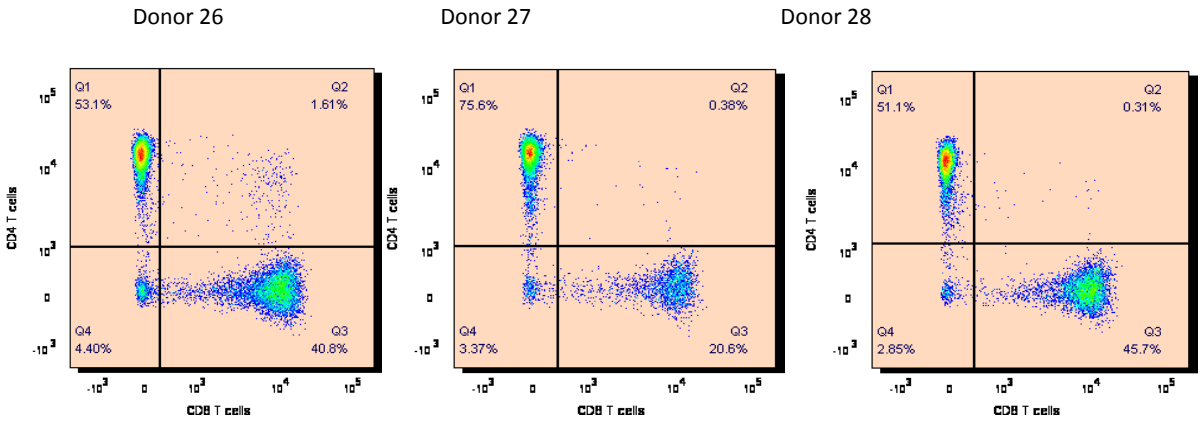


Figure 4.2: CD4+/CD8+ distribution on day 0 for donor cones 26, 27, 28

BATCH 1 (+CD3/8)

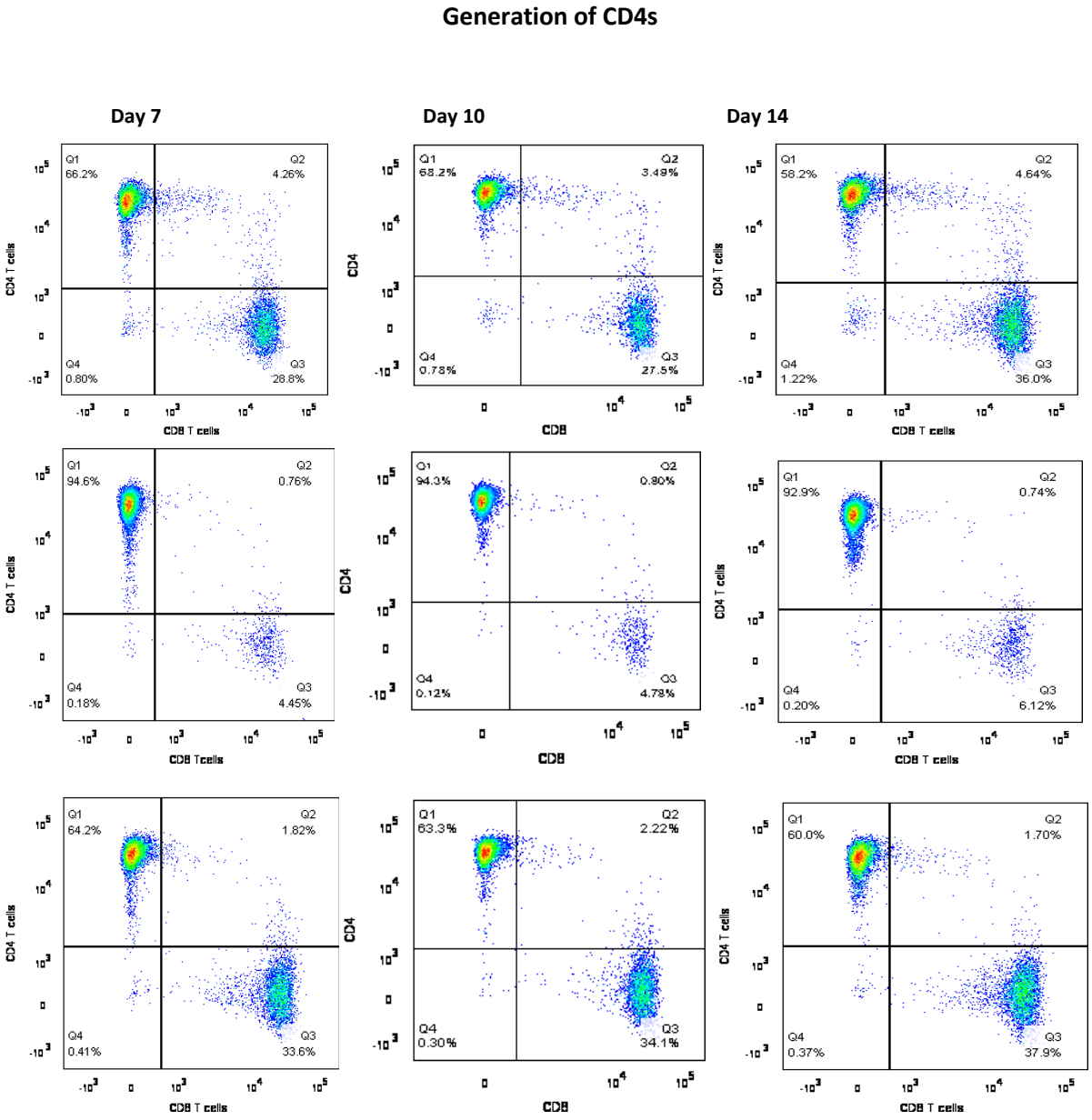


Figure 4.3: Day 7, 10 and 14 CD4+ cultures shown for donors using batch 1 of the CD3/8+ bispecifics

BATCH 1 (+CD3/4)

Generation of CD8s

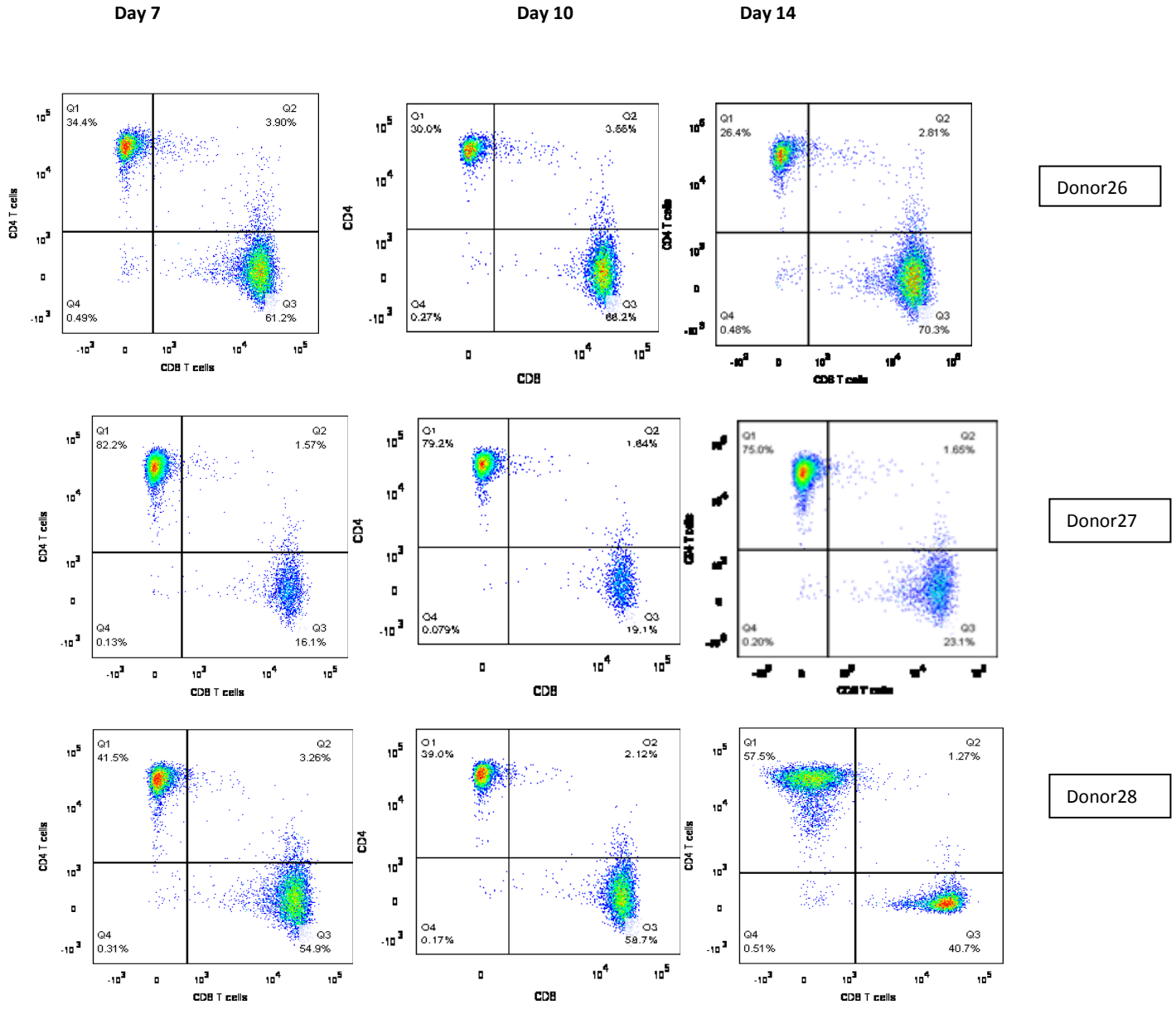


Figure 4.4: Day 7, 10 and 14 CD8+ cultures shown for donors using batch 1 of the CD3/4+ bispecifics

BATCH 2 (+CD3/8)

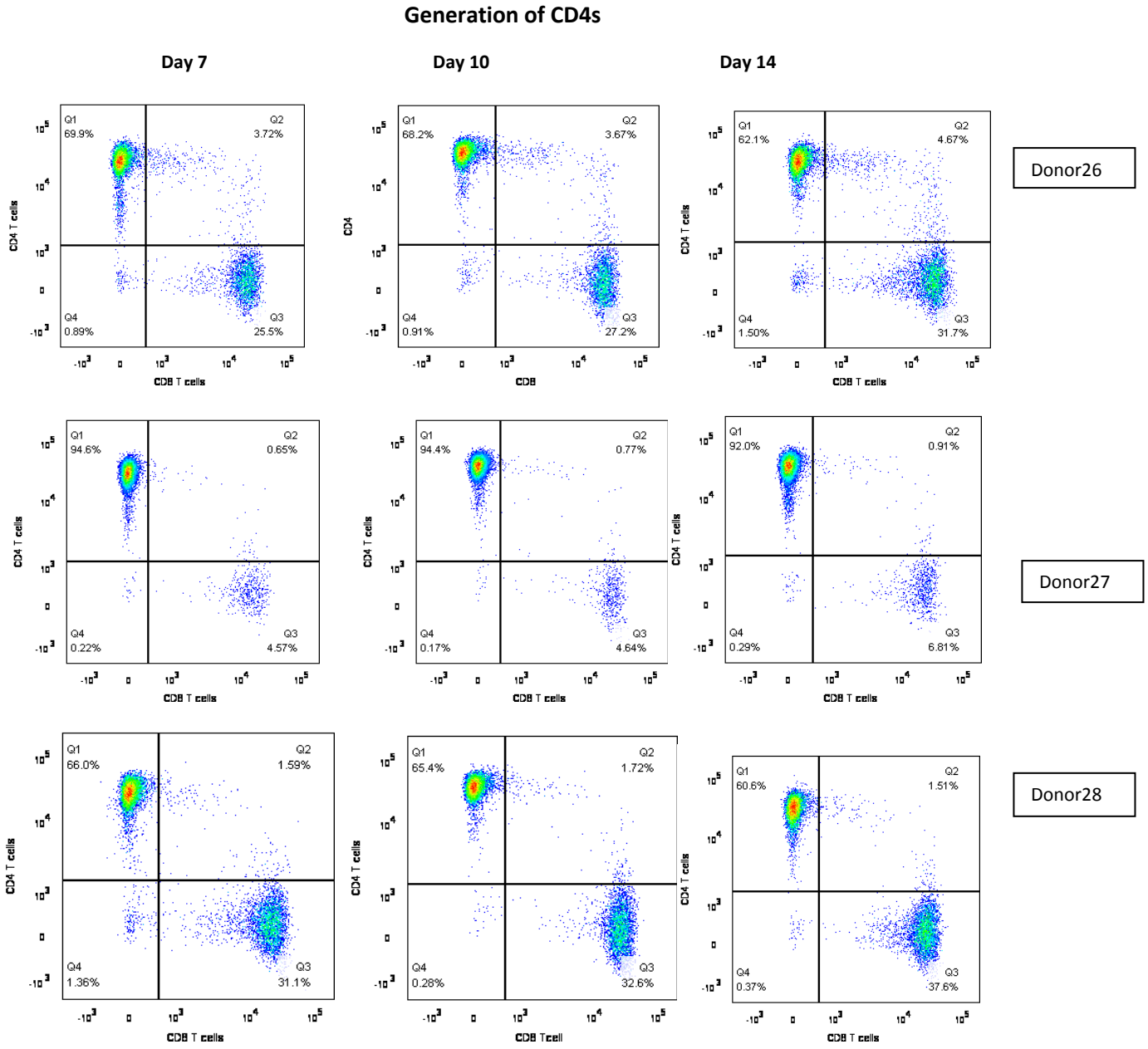


Figure 4.5: Day 7, 10 and 14 CD4+ cultures shown for donors using batch 2 of the CD3/8+ bispecifics

BATCH 2 (+CD3/4)

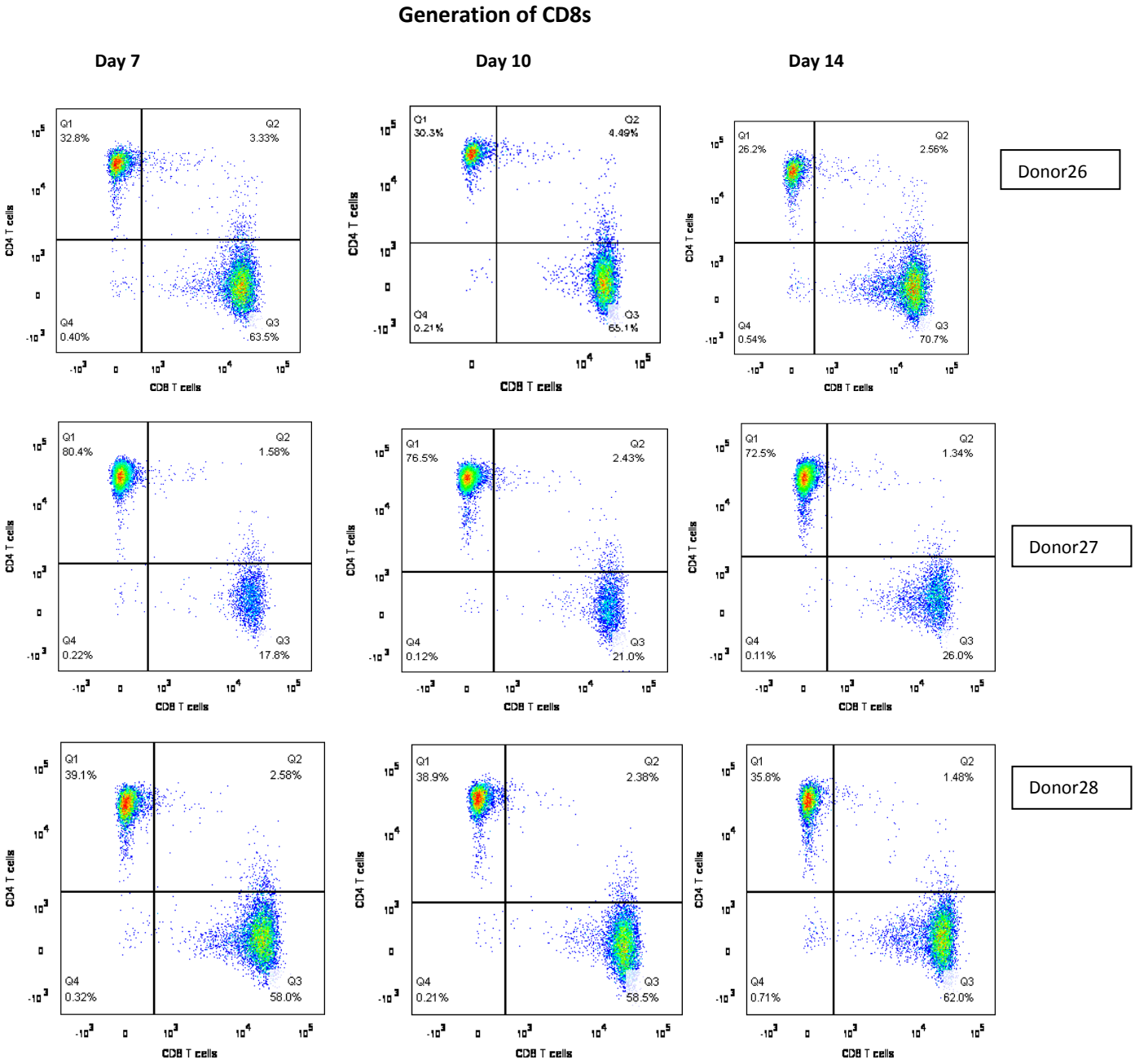


Figure 4.6: Day 7, 10 and 14 CD8+ cultures shown for donors using batch 2 of the CD3/4+ bispecifics

Table 4.7 showing CD4+ and CD8+ purities at days 0, 7, 10, 14 post expansion

Batch 1 (+ CD3/8)
CD4s

	Day 0	Day 7	Day 10	Day 14
26	53.1%	66.2%	68.2%	58.2%
27	75.6%	94.6%	94.3%	92.9%
28	51.1%	64.2%	63.3%	60%

Batch 1 (+ CD3/4)
CD8s

	Day 0	Day 7	Day 10	Day 14
26	40.8%	61.2%	66.2%	70.3%
27	20.6%	16.1%	19.1%	23.1%
28	45.7%	54.9%	58.7%	40.7%

Batch 2 (+ CD3/8)
CD4s

	Day 0	Day 7	Day 10	Day 14
26	53.1%	69.9%	68.2%	62.1%
27	75.6%	94.6%	94.4%	92 %
28	51.1%	66 %	65.4%	60.6%

Batch 2 (+ CD3/4)
CD8s

	Day 0	Day 7	Day 10	Day 14
26	40.8%	63.5%	65.1%	70.7%
27	20.6%	17.8%	21 %	26 %
28	45.7%	58 %	58.5%	62 %

Table 4.8 showing expansion of CD4+ and CD8+ populations at days 0, 7, 10, 14

Batch 1 (+ CD3/8)
CD4s

	Day 0	Day 7	Day 10	Day 14
26	$1x10^6$	$7.2x10^6$	$9.15 x10^6$	$9.15x10^6$
27	$1.5x10^6$	$11.25x10^6$	$13.65x10^6$	$8.25x10^6$
28	$1.5x10^6$	$8.25x10^6$	$9.45x10^6$	$13.05x10^6$

Batch 1 (+ CD3/4)
CD8s

	Day 0	Day 7	Day 10	Day 14
26	$1x10^6$	$9.9 x10^6$	$10.5x10^6$	$12.9x10^6$
27	$1.5x10^6$	$11.55x10^6$	$12.6x10^6$	$10.05x10^6$
28	$1.5x10^6$	$7.8x10^6$	$8.55x10^6$	$12x10^6$

Batch 2 (+ CD3/8)
CD4s

	Day 0	Day 7	Day 10	Day 14
26	$1x10^6$	$8.4x10^6$	$7.8x10^6$	$11.4x10^6$
27	$1.5x10^6$	$10.35x10^6$	$9.75x10^6$	$10.8x10^6$
28	$1.5x10^6$	$8.7x10^6$	$10.35x10^6$	$18x10^6$

Batch 2 (+ CD3/4)
CD8s

	Day 0	Day 7	Day 10	Day 14
26	$1x10^6$	$8.4x10^6$	$11.1x10^6$	$9x10^6$
27	$1.5x10^6$	$11.25x10^6$	$10.35x10^6$	$6.9x10^6$
28	$1.5x10^6$	$10.65x10^6$	$10.95x10^6$	$21x10^6$

Appendix 5: Toxicity tables Adapted from the FDA

Table 5.1 Laboratory parameters

PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 EXTREME
HAEMATOLOGY				
Hb	10.0-10.9 g/dL	9.0-9.9 g/dL	7.0-8.9 g/dL	<7.0 g/dL
White Blood Count	13.0 – 14.9 $x10^9$/l or 2 – 2.5 $x10^9$/l	15.0 – 19.9 $x10^9$/l or 1.5 – <2.0 $x10^9$/l	20.0 – 29.9 $x10^9$/l or 1 – <1.5 $x10^9$/l	≥30.0 or <1.0 $x10^9$/l
Absolute Neutrophils	1.3-1.0 $x10^9$/l	<1.0-≥0.75 $x10^9$/l	<0.75-≥0.5 $x10^9$/l	<0.5 $x10^9$/l
Percent neutrophils	>80%	90%	≥95%	---
Lymphocytes	0.7- 0.899 $x10^9$/l	0.5-0.699 $x10^9$/l	0.35-0.499 $x10^9$/l	<0.35 $x10^9$/l
Platelets	100 –124.999 x 10^9/l	50 – 99.999 x 10^9/l	25 – 49.999 $x10^9$/l	<25.0 $x10^9$/l
CD4+ Count	300-400/mm^3	<300mm^3	<200/mm^3	<100/mm^3
BIOCHEMISTRY				
Potassium				
Hyperkalemia	5.6 – 6.0 meq/L	6.1-6.5 meq/L	6.6-7.0 meq/L	>7.0 meq/L
Hypokalemia	3.0 – 3.4 meq/L	2.5 – 2.9 meq/L	2.0– 2.4 meq/L	<2.0 meq/L
Bilirubin				
Hyperbilirubinemia	>1.25 – 2.0 x ULN	>2.0 – 2.5 x ULN	>2.5 – 5 x ULN	>5 x ULN
Glucose				
Hypoglycaemia	2.3-2.4 mmol/l	2.1-2.2 mmol/l	1.5-2.0 mmol/l	<1.5 mmol/l
Hyperglycaemia nonfasting; no prior diabetes	7.0-10.0 mmol/l	10.1-15.0 mmol/l	15.1-25.0 mmol/l	>25.0 mmol/l
Transaminases				
AST (SGOT)	1.25 – 2.5 x ULN	>2.5 – 5.0 x ULN	>5.0 – 10.0 x ULN	> 10.0 x ULN
ALT (SGPT)	1.25 – 2.5 x ULN	>2.5 – 5.0 x ULN	>5.0 – 10.0 x ULN	> 10.0 x ULN
GGT	1.25 – 2.5 x ULN	>2.5 – 5.0 x ULN	>5.0 – 10.0 x ULN	> 10.0 x ULN
Alk Phos	1.25 – 2.5 x ULN	>2.5 – 5.0 x ULN	>5.0 – 10.0 x ULN	> 10.0 x ULN
Amylase	>1.0 – 1.5 x ULN	>1.5 – 2.0 x ULN	>2.0 – 5.0 x ULN	>5.0 x ULN
Creatinine	130-180μmol/l	181-360μmol/l	361-720μmol/l	>720μmol/l
URINALYSIS				
Proteinuria: 24 hour urine	200 mg - 1 g loss/day OR <0.3% OR <3 g/l	1 – 2 g loss/day OR 0.3 – 1.0% OR 3 - 10 g/l	2 – 3.5 g loss/day OR >1.0% OR > 10 g/l	Nephrotic syndrome OR >3.5 g loss/day
Haematuria	Microscopic only ≤10 RBC/HPF	>10 RBC/HPF	Gross, with or without clots OR RBC casts	Obstructive OR transfusion req

Table 5.2: Solicited vaccine reactions

GENERAL				
Fever Oral>12 hours	37.7 - 38.9°C (100.0 – 101.5°F)	39.0 – 39.7°C (101.6 – 102.9°F)	39.8 – 40.5°C (103 - 105°F)	>40.5°C (105°F) OR max temp of >105°F
Chills/rigors	Mild hot/cold flush requires blanket or occasional over the counter treatment	Limiting daily activity >6 hours, or need regular over the counter treatment	Uncontrollable shaking, prescription treatment needed	Hospitalisation
Malaise/abnormal tiredness	Normal activity reduced – not bad enough to go to bed	Fatigue such that ½ day in bed for 1 or 2 days	Fatigue such that in bed all day or ½ day for more than 2 days	Hospitalisation
General (all over) muscle aches and pains	No limitation of activity	Muscle tenderness, aches/pains limiting activity e.g. difficulty climbing stairs	Severe limitation e.g. can't climb stairs	Hospitalisation
Headache	No treatment or responds to over the counter treatment	Regular over the counter or occasional prescription treatment needed	Regular prescription treatment needed	Hospitalisation
Nausea	Intake maintained	Intake reduced less than 3 days	Minimal intake 3 days or more	Hospitalisation
CUTANEOUS				
Discomfort/pain in injected muscle (including ache) or overlying skin	Mild itch or ache that responds to over the counter treatment, if needed	Pain requiring regular over the counter treatment or occasional prescription treatment	Pain requiring regular prescription treatment	Hospitalisation
Immediate reactions (within 6 hours of injection)	Symptoms of irritation locally (usually itching at the injection site) OR Erythema +/- swelling at the injection site		Laryngeal oedema insufficient to require intubation; diarrhoea insufficient to require IV fluids, or asthma insufficient to require hospitalisation OR Urticaria, angio-oedema OR Generalised pruritus	Anaphylactic shock
Erythema at injection site	Erythema up to and including 50% of baseline arm or thigh circumference OR Symptoms of irritation that are easily tolerated and do not require repeated medication OR Both	Erythema greater than 50% of the arm or thigh circumference at baseline With or without Symptoms of irritation that do not require repeated medication OR Symptoms of irritation that require repeated medication AND erythema up to and	Erythema greater than 50% of the arm or thigh circumference at baseline AND symptoms of irritation requiring repeated medication	Hospitalisation

		including 50%		
Blistering or ulceration at injection site	Fluid filled vesicles or superficial disruption of epithelium covering an area < 1cm	Fluid filled vesicles or superficial disruption of epithelium, area 1 - 2cm OR Blood filled vesicles OR Full thickness disruption of epithelium healed within 2 weeks	Full thickness disruption of epithelium not healed within 2 weeks	**Necrosis**
CUTANEOUS				
Soft swelling – local	Swelling <25% of arm or thigh	Swelling 25-50% of arm or thigh	Swelling >50% of arm or thigh Or Induration/hardened swelling (when considered by the clinician to be associated with a process arising in the muscle)	

Appendix 6: CUTHIVAC 001 ICS data

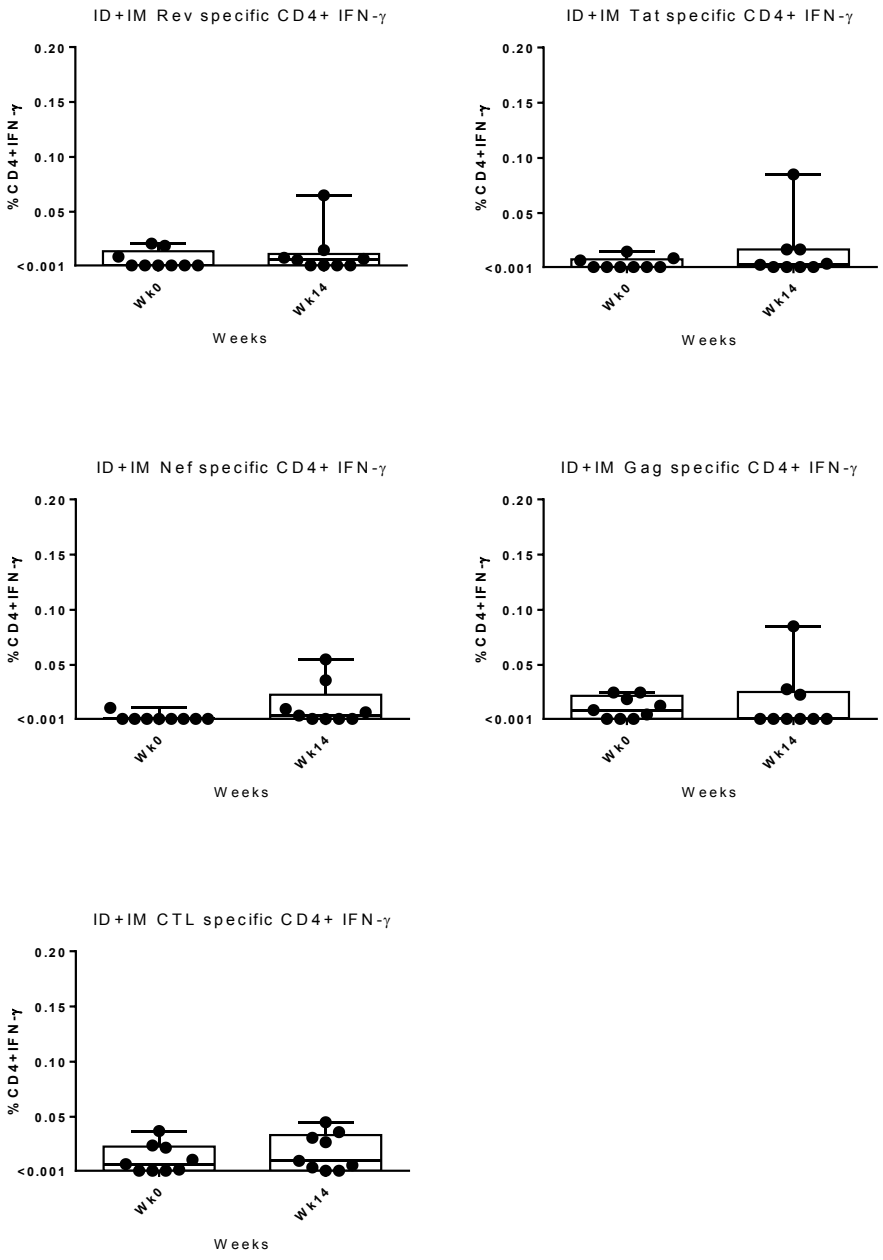


Figure 6.1: CD4+ IFN-ɣ ICS data for ID+IM group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

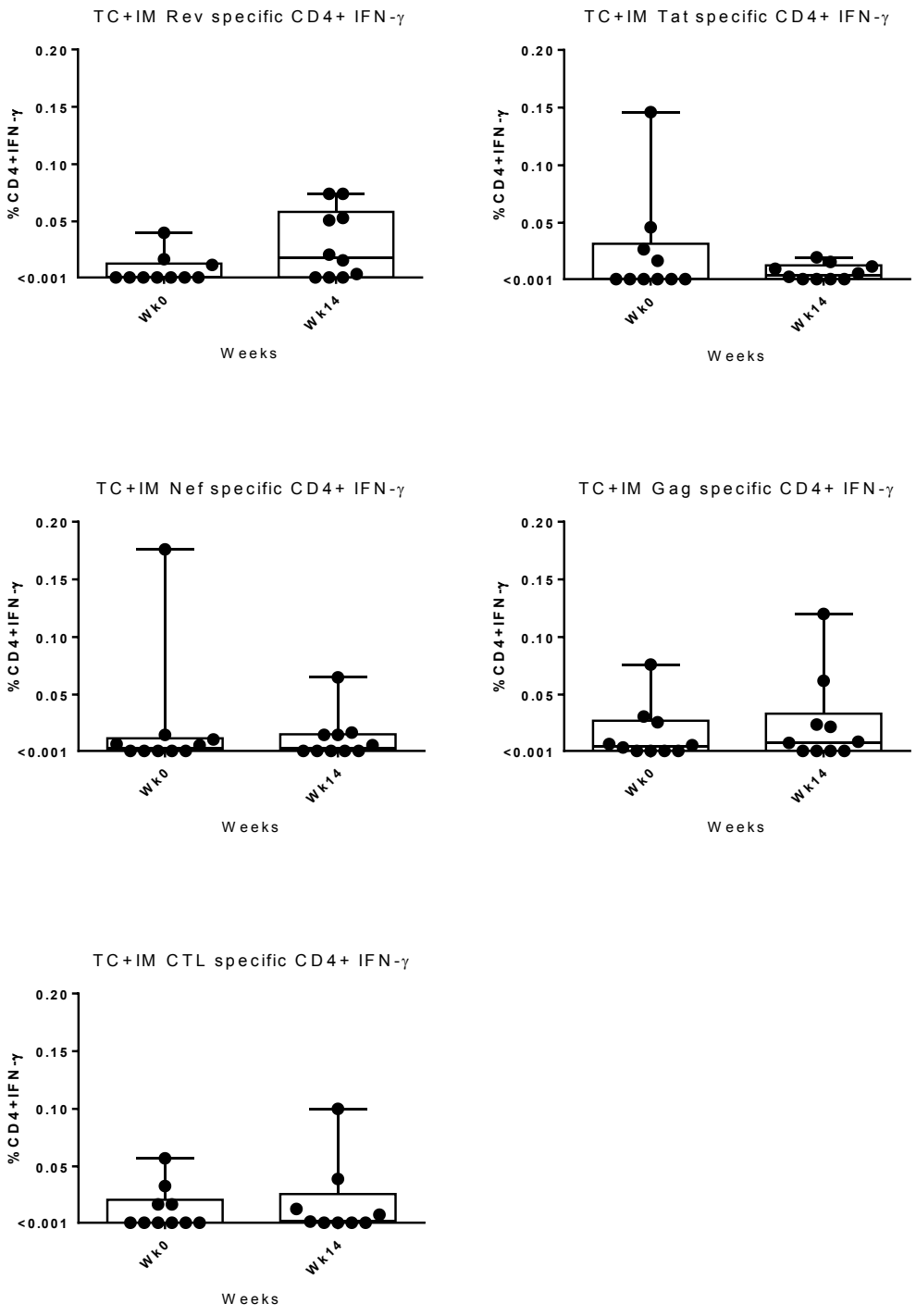


Figure 6.2: CD4+ IFN-ɣ ICS data for TC+IM group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

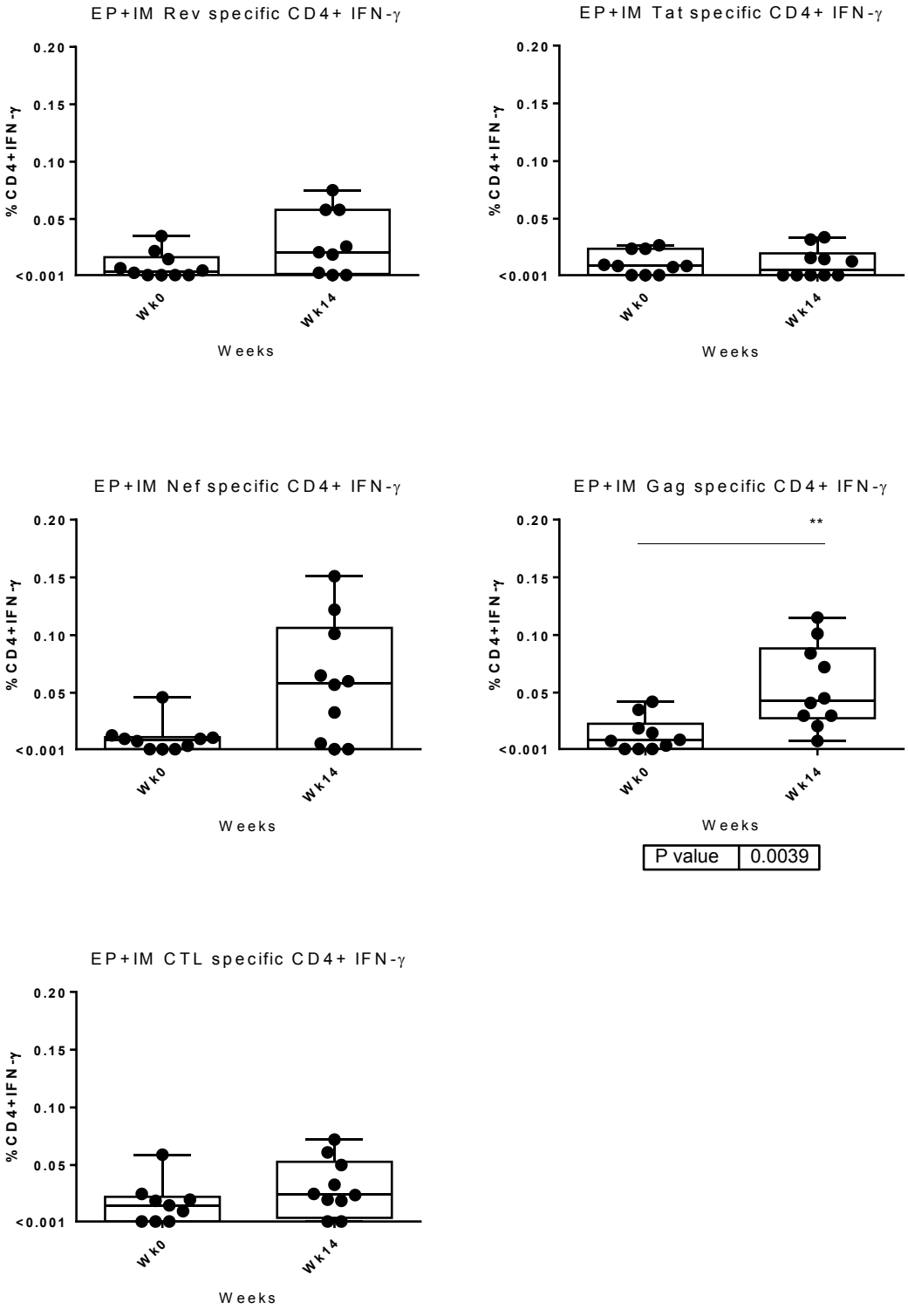


Figure 6.3: CD4+ IFN-ɣ ICS data for EP+IM group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

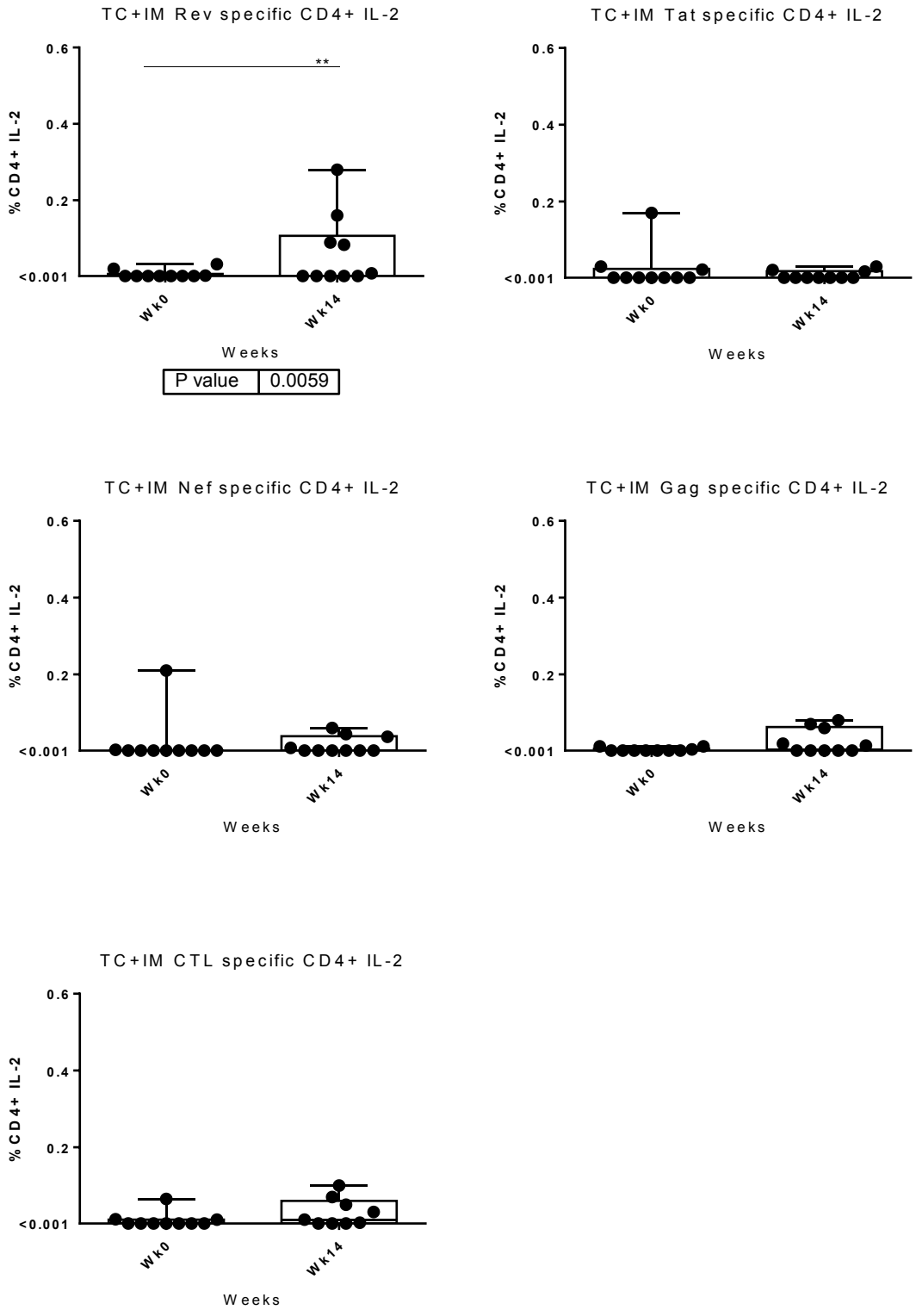


Figure 6.4: CD4+ IL-2 ICS data for TC+IM group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

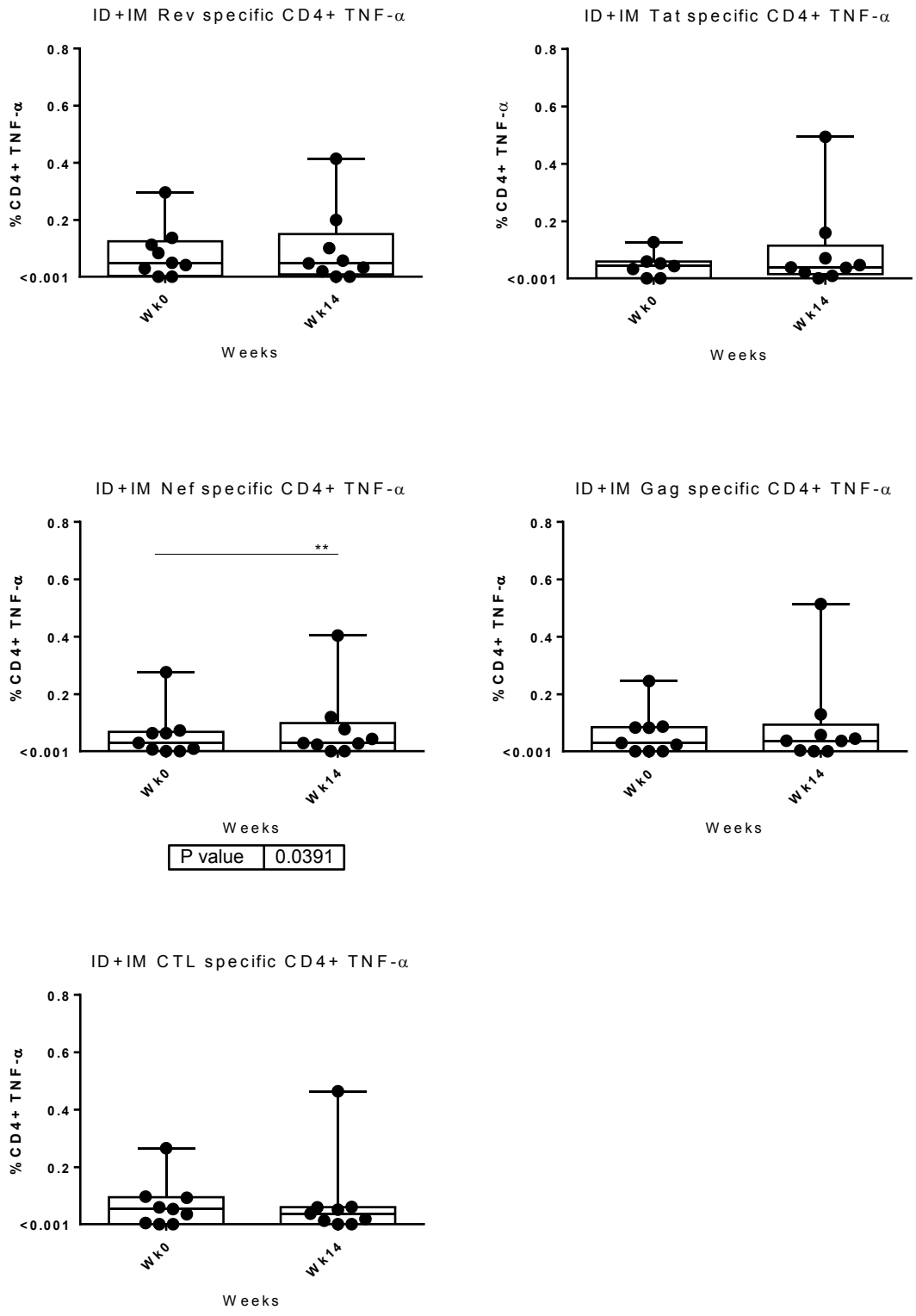


Figure 6.5: CD4+ TNF-α ICS data for ID+IM group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

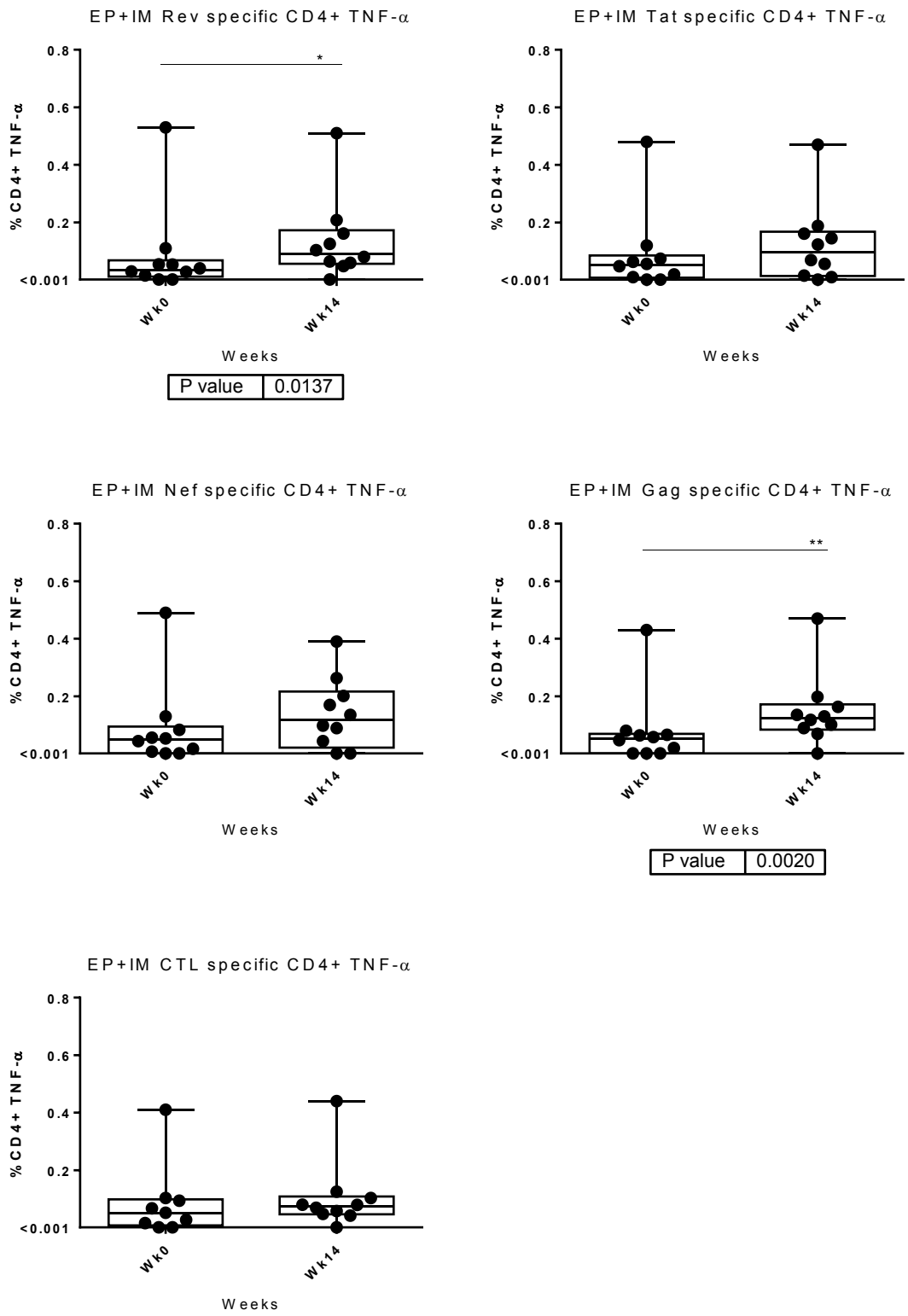
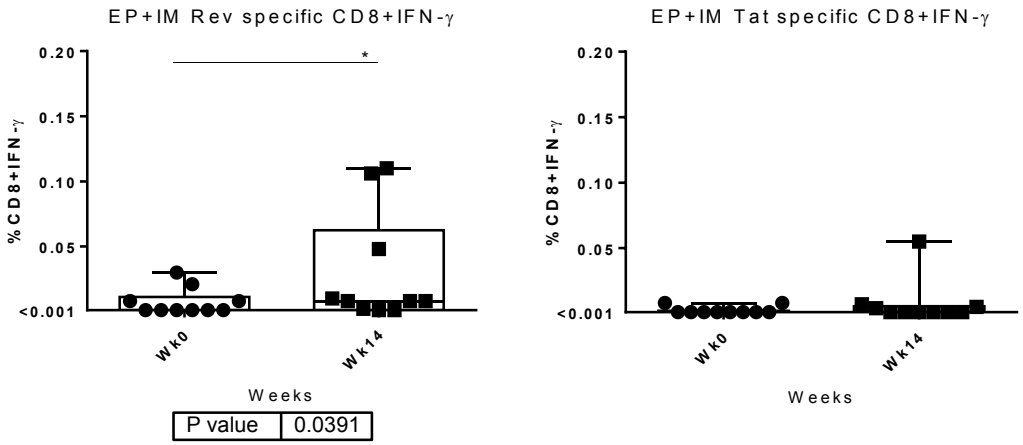
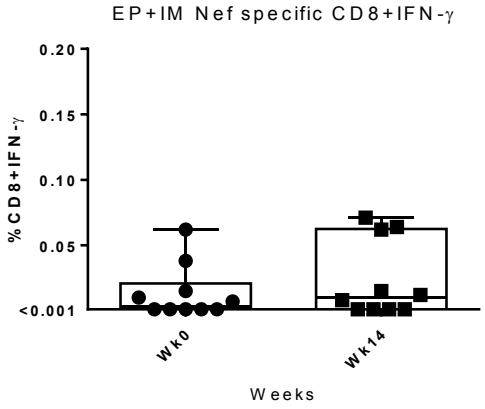
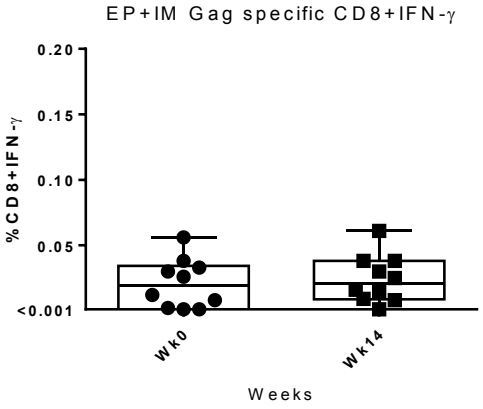


Figure 6.6: CD4+ TNF-α ICS data for EP+IM group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.







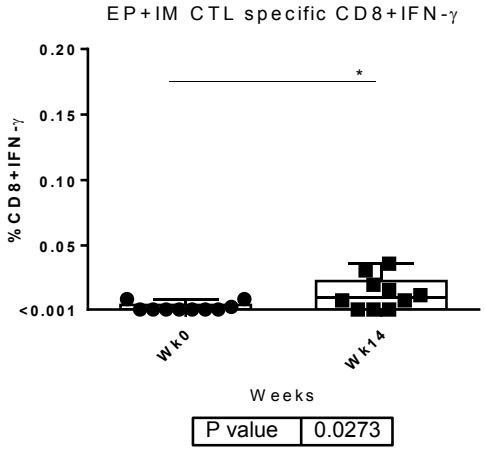


Figure 6.7: CD8+ IFN-ɣ ICS data for EP+IM group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

Appendix 7: CUTHIVTHER 001 ICS data

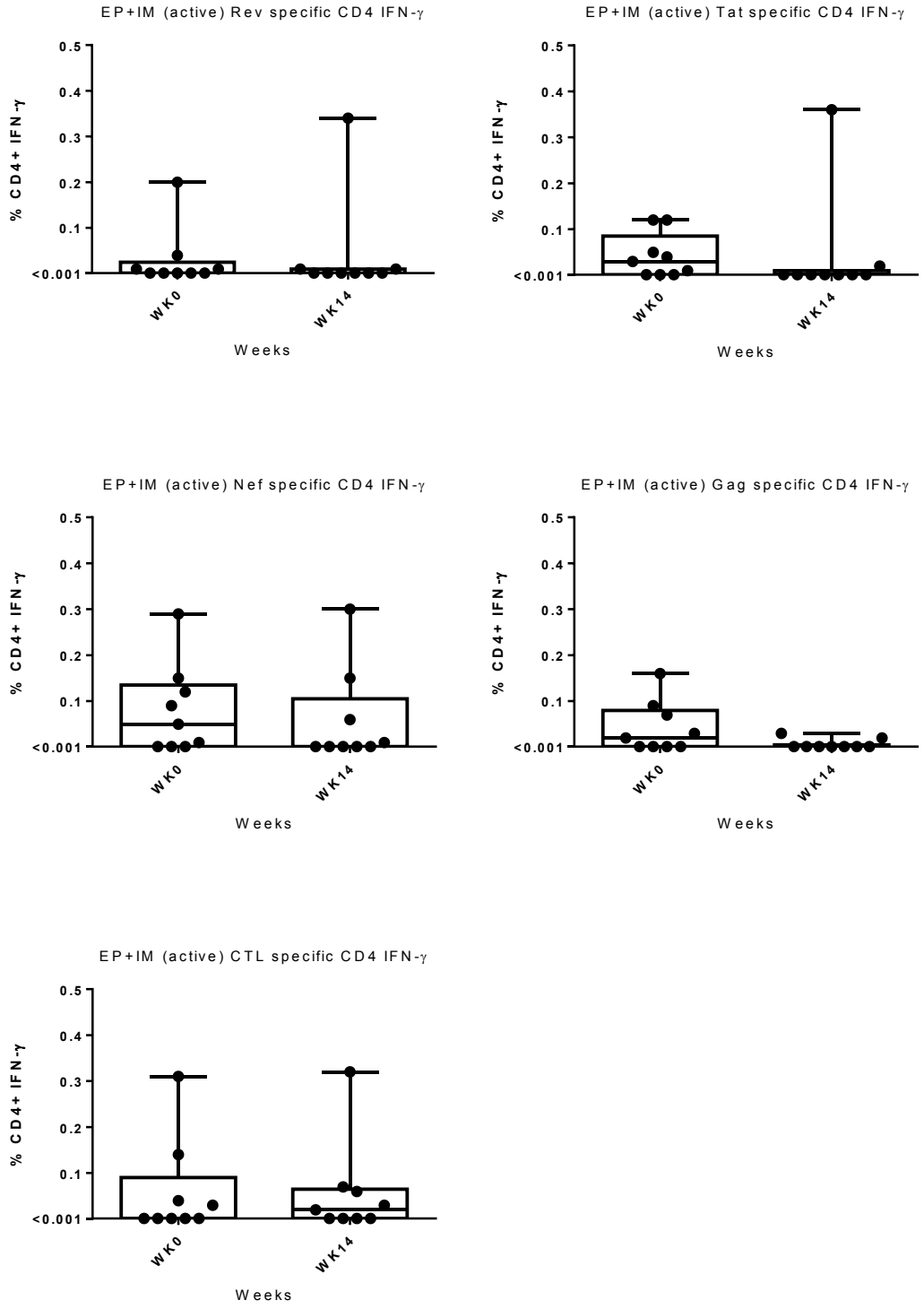


Figure 7.1: CD4+ IFN-ɣ ICS data for EP+IM (active) group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

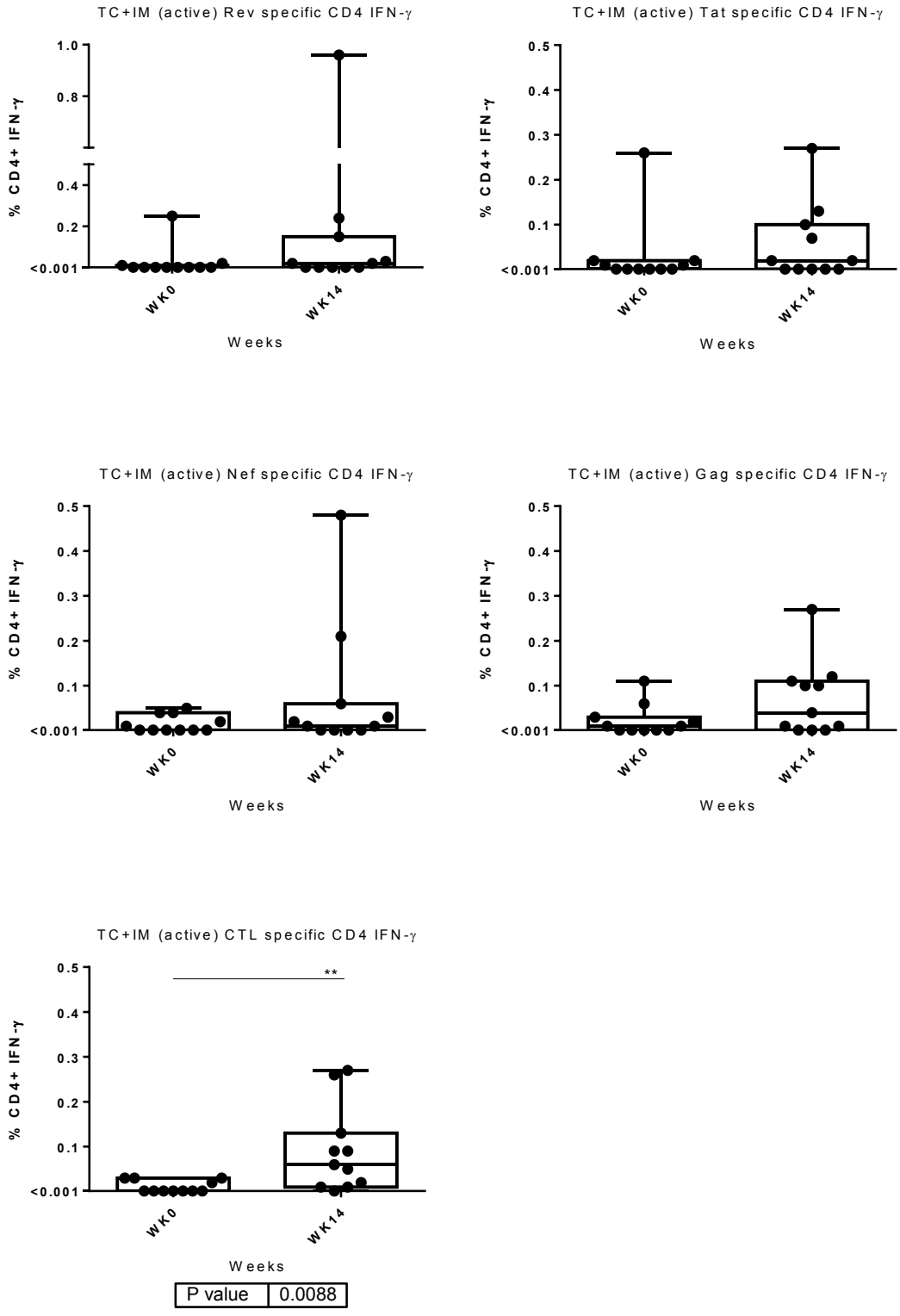


Figure 7.2: CD4+ IFN-ɣ ICS data for TC+IM (active) group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

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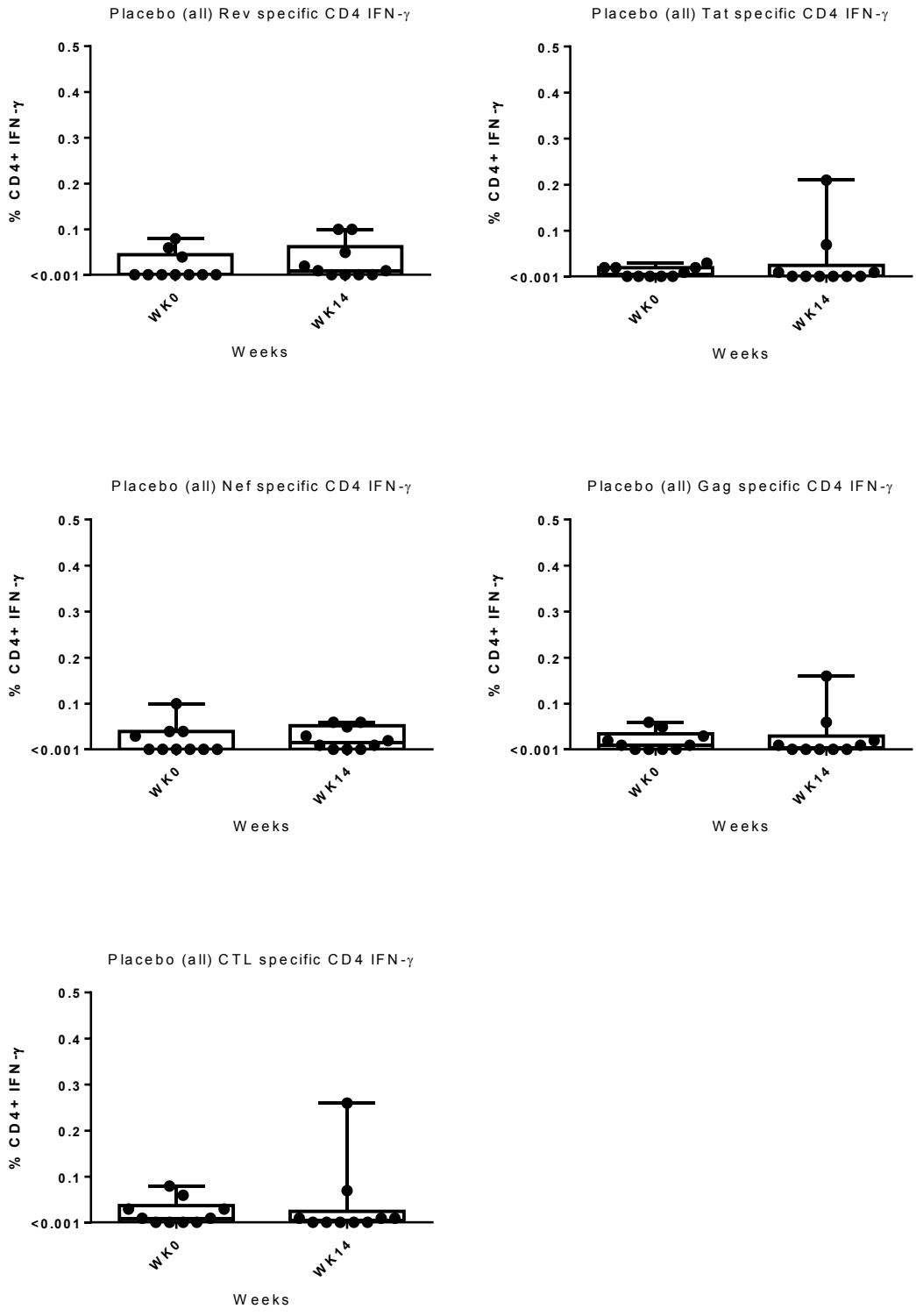

Figure 7.3: CD4+ IFN-ɣ ICS data for placebo group (all) comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

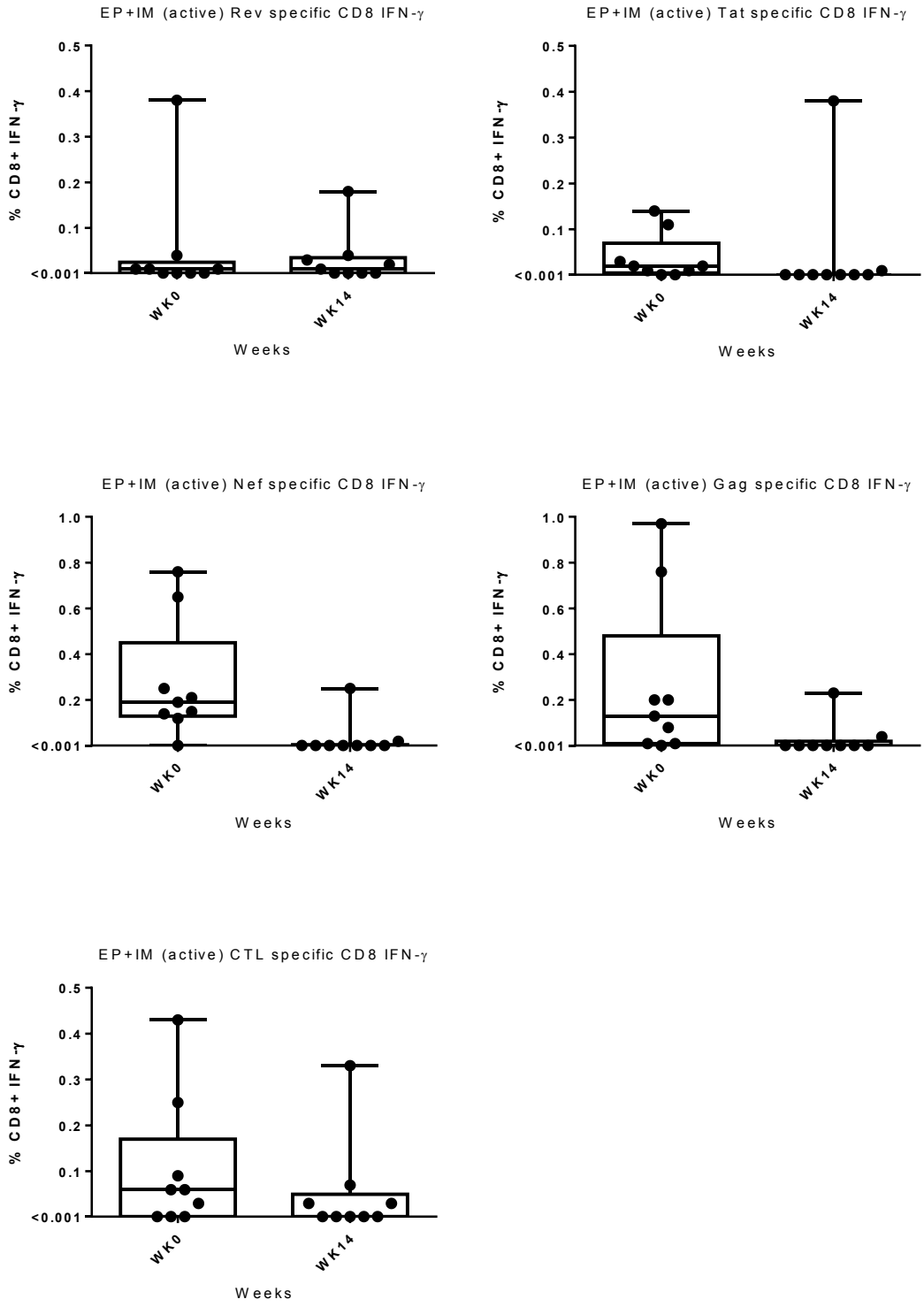

Figure 7.4: CD8+ IFN-ɣ ICS data for EP+IM (active) group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

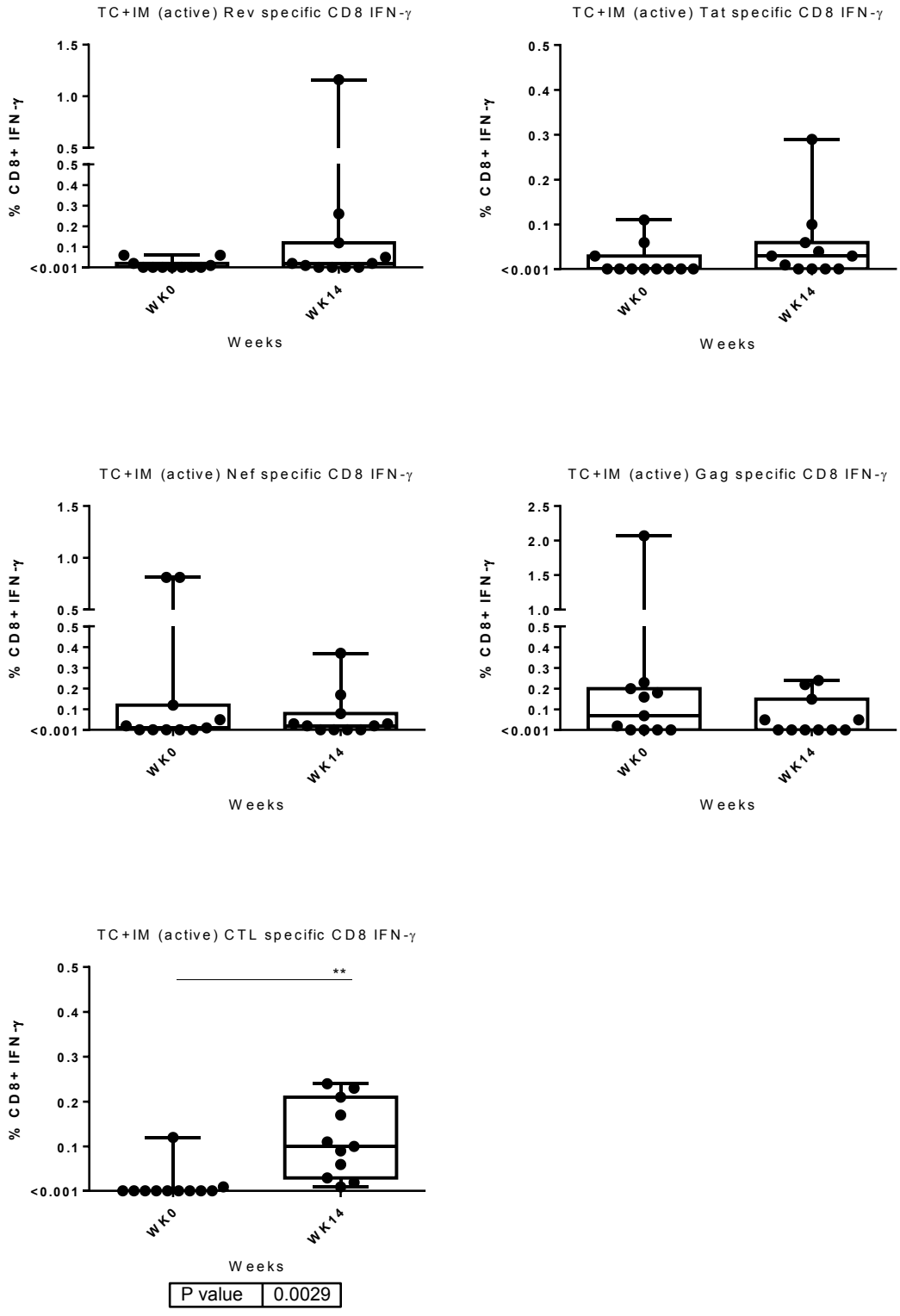

Figure 7.5: CD8+ IFN-ɣ ICS data for TC+IM (active) group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

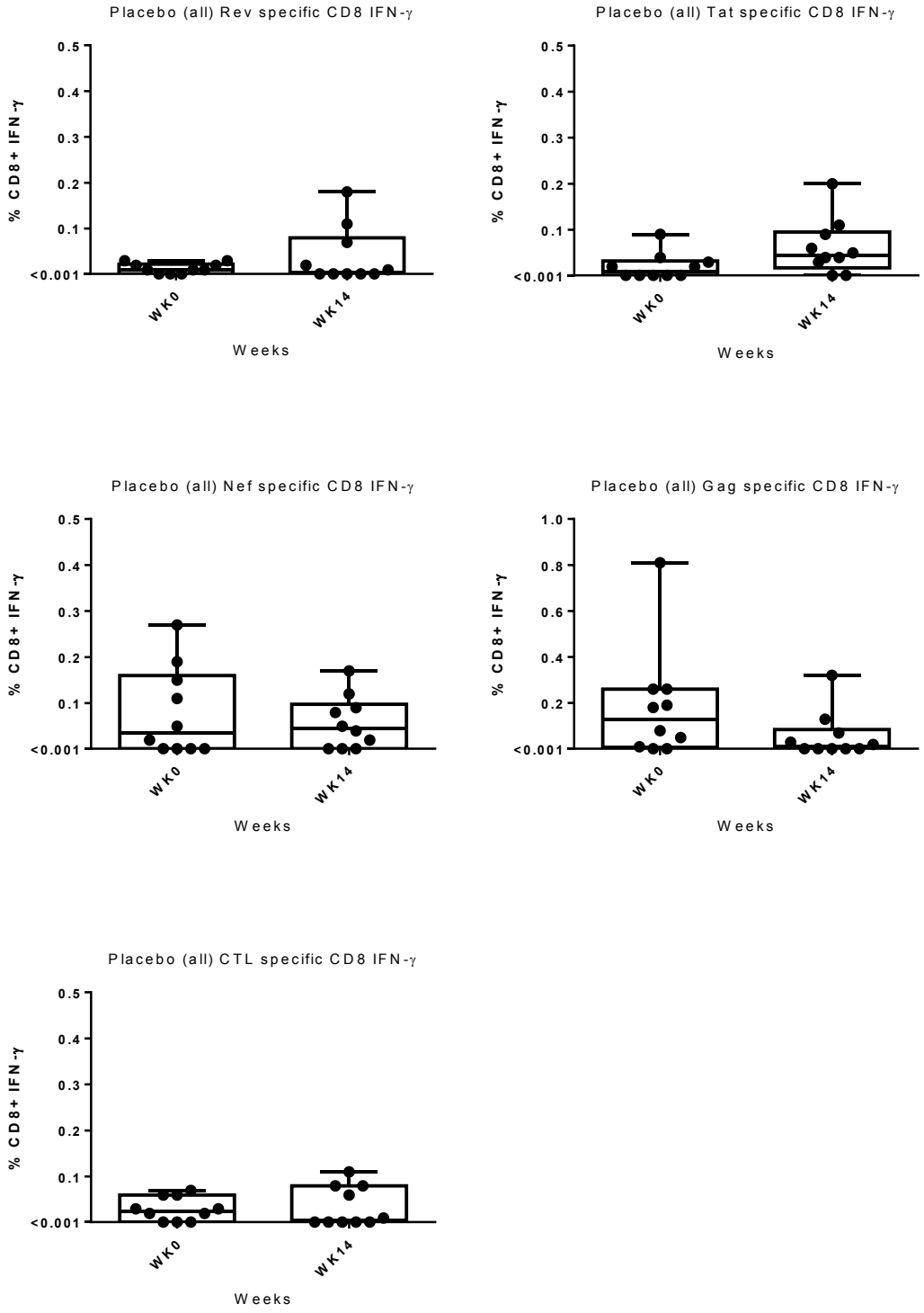

Figure 7.6: CD8+ IFN-ɣ ICS data for placebo group (all) comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

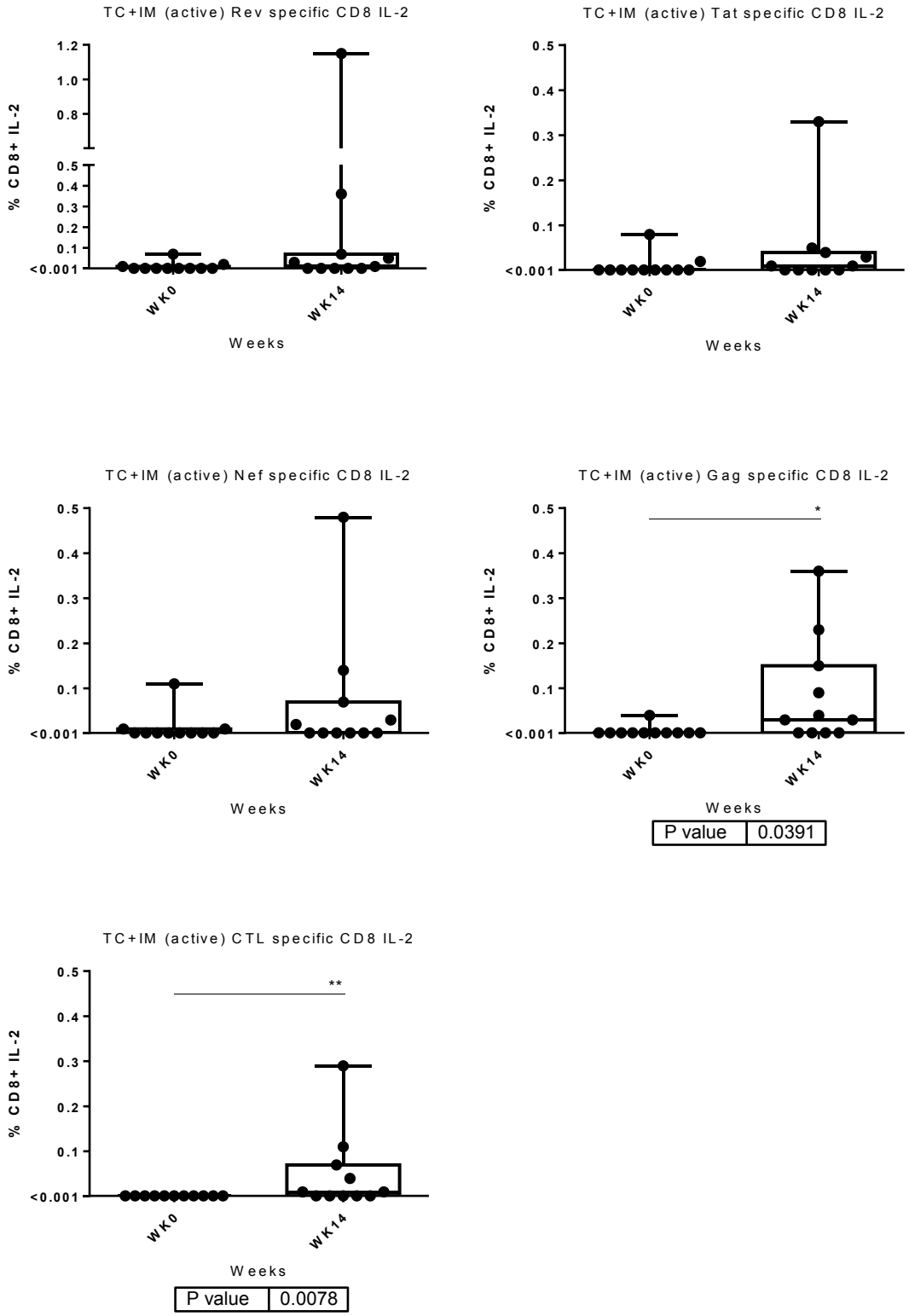

Figure 7.7: CD8+ IL-2 ICS data for TC+IM (active) group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

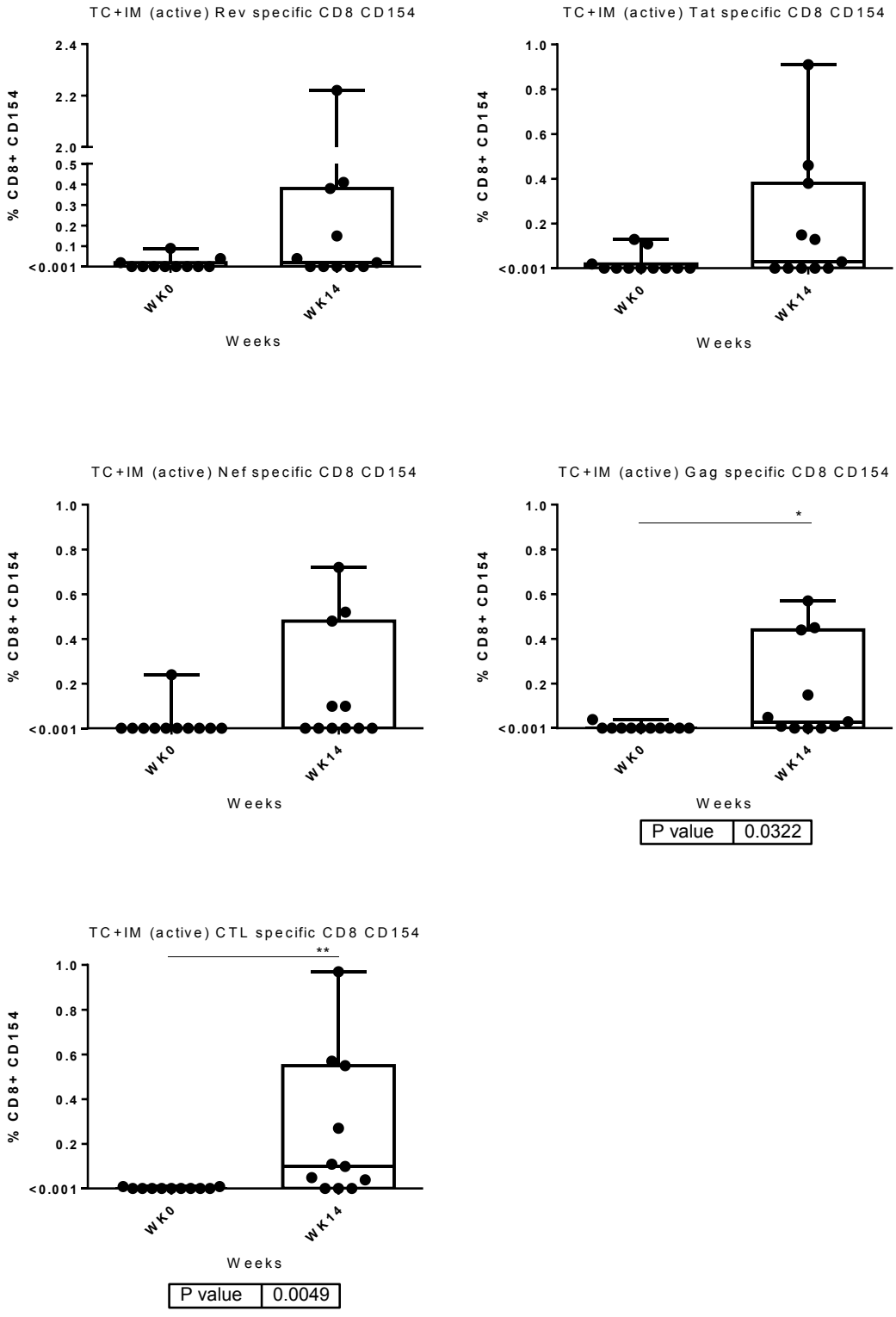

Figure 7.8: CD8+ CD154 ICS data for TC+IM (active) group comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.

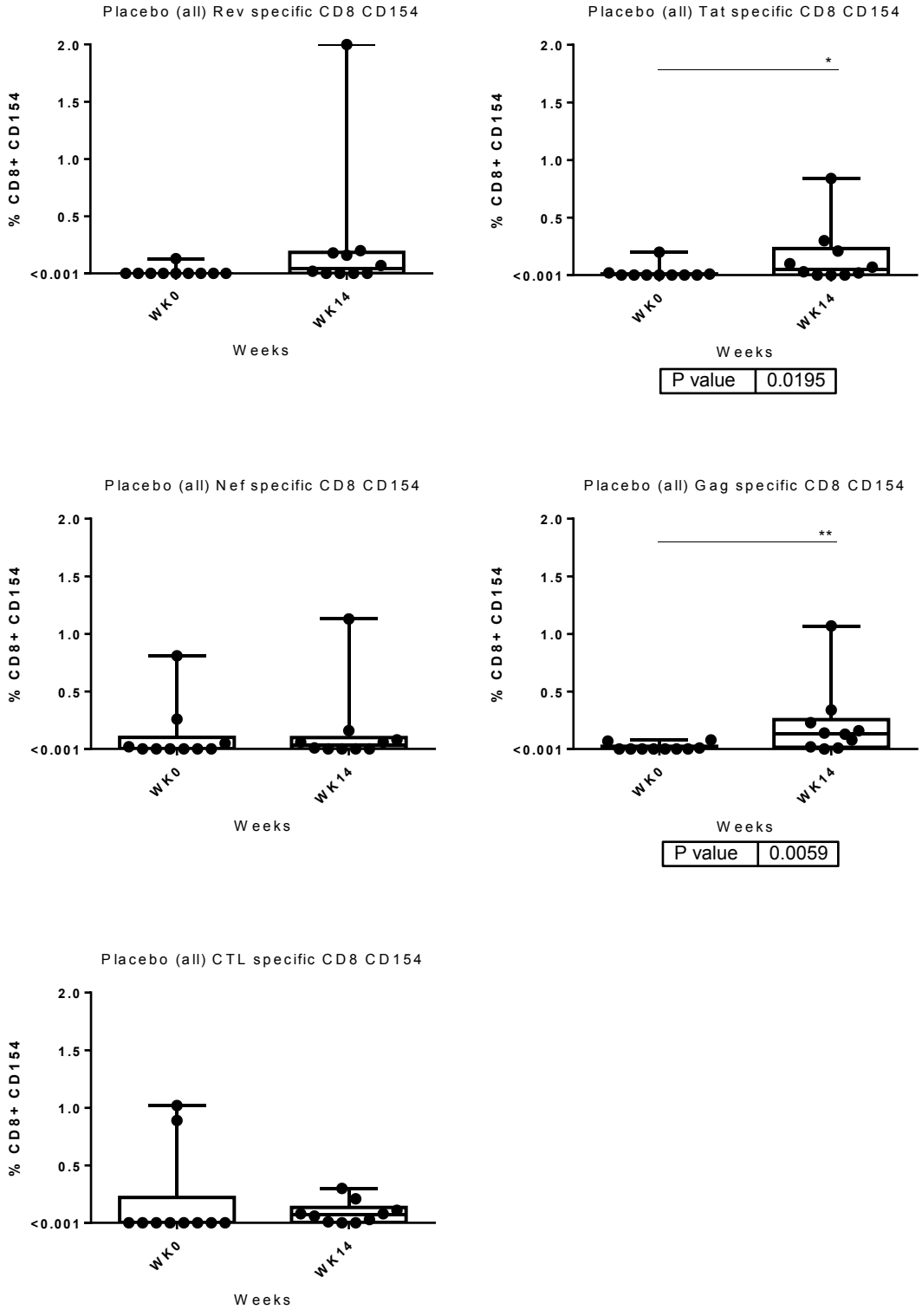

Figure 7.9: CD8+ CD154 ICS data for placebo group (all) comparing week 0 (pre- vaccination) to week 14 (primary end point). P values shown where appropriate with statistical significance set at p=<0.05.