Cytomegalovirus Modulation of the Immune System in ANCA Associated Vasculitis

by

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Abstract

Infection and cardiovascular disease represent the two most important sources of mortality in ANCA associated vasculitis (AAV). Expansions of CD4+CD28null T-cells that are only present in cytomegalovirus (CMV) positive individuals have previously been associated with increased infection and mortality in AAV, and cardiovascular disease in other inflammatory diseases.

The work described in this thesis examines the hypothesis that subclinical CMV reactivation in AAV drives the expansion of CD4+CD28null T-cells thereby leading to the observed adverse outcomes. To investigate this, a proof of concept clinical trial of 6 months valaciclovir treatment or no additional therapy was designed and implemented in CMV seropositive AAV patients in remission.

Valaciclovir treatment successfully blocked CMV reactivation and in turn this led to a reduction in the proportion of CD4+CD28null T-cells in the treated patients together with favourable changes in other associated CMV induced changes on the immune system. CD4+CD28null T-cells in AAV were identified as Th1, proinflammatory cytotoxic T-cells, able to target endothelial cells and were independently associated with increased arterial stiffness, an established marker of cardiovascular risk.

These findings implicate subclinical CMV reactivation as a potentially reversible cause of vascular pathology in inflammatory disease and open novel therapeutic opportunities.

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Table of Contents

Page

Chapter 1 Introduction 2

1.1 ANCA associated vasculitis 2
1.1.1 Definitions and nomenclature 2
1.1.2 Epidemiology of AAV 4
1.1.3 Clinical Features of AAV 4
1.1.4 AAV pathophysiology 6
1.1.4.1 Involvement of T-cell immunity in AAV pathophysiology 8
1.1.5 Treatment of AAV 10
1.1.5.1 Induction of remission in AAV 11
1.1.5.2 Maintenance of remission in AAV 14
1.1.5.3 Treatment of refractory AAV disease 15
1.1.5.4 Current treatment recommendations for AAV 16
1.1.6 Morbidity and mortality in AAV 18
1.1.6.1 Prognosis 18
1.1.6.2 Infection and cardiovascular disease in AAV 18
1.1.6.3 Dysregulated T-cell immunity contributes to AAV morbidity 20
1.2 CD4+CD28null T-cells 21
1.2.1 Organisation of the immune system 21
1.2.2 CD4 T-cells and their subsets 22
1.2.2.1 T-cell activation 22
1.2.2.2 CD4 T-cell memory differentiation 24
1.2.3 CD28 expression and downregulation 26
1.2.4 Characteristics of CD4+CD28null T-cells 26
1.2.5 CD4+CD28null T-cells are associated with inflammation 29
1.2.5.1 CD4+CD28null T-cell expansion in inflammatory disease states 30
1.2.5.2 CD4+CD28null T-cells in AAV 31
1.2.5.3 CD4+CD28null T-cells in renal disease 31
1.2.5.4 CD4+CD28null T-cells in cardiovascular disease 33
1.2.6 Origin of CD4+CD28null T-cells 34

1.3 Cytomegalovirus 37
1.3.1 CMV epidemiology 37
1.3.2 CMV infection and the host response 37
1.3.3 CMV leads to systemic dysregulation of the immune system 39
1.3.4 CMV is associated with inflammation and vascular pathology 40
1.3.5 CMV is associated with immunosenescence 42
1.3.5.1 CMV and immunological functional exhaustion 43
1.3.6 CMV replication in latency and pharmacological suppression 46
1.4 Summary and hypothesis 48
1.4.1 Thesis aims and objectives 50

Chapter 2 Methods 52

2.1 Study Design Overview 52
2.2 Clinical Trial Arm Design and Implementation 53
2.3 Valaciclovir to prevent Cytomegalovirus (CMV) mediated adverse modulation of the immune system in ANCA associated VAScultis (CANVAS): Study protocol for a randomised controlled clinical trial 55
2.3.1 Design 55
2.3.2 Participants 58
2.3.3 Schedule of Assessments 60
2.3.4 Study Assessments 63
2.3.5 Randomisation 64
2.3.6 Treatment 65
2.3.7 Sample Size Calculation and Planned Statistical Analyses 66
2.3.8 Trial Management and Monitoring 67
2.3.9 Funding Declaration 69
2.3.10 Sponsor 69
2.4 Clinical Trial Sample Flow and Analysis 70
2.5 Cross-Sectional Arm Design 71
2.5.1 Schedule of assessments 72
2.6 Laboratory Assays 73

2.6.1 Viral load quantitation 73
2.6.2 Whole blood surface staining of lymphocytes 78
2.6.3 Measuring soluble markers of inflammation and endothelial damage 83
2.6.4 Peripheral blood mononuclear cell isolation 86
2.6.5 Peripheral blood mononuclear cell stimulation 87
2.6.6 CMV lysate production 90
2.6.7 CD4+CD28null percentage tracking experiments 93
2.6.8 Estimation of CMV IgG titre 95
2.7 Formal Assay Validation Analyses 98
2.7.1 Surface Staining of CD3 CD4 CD28 in Whole Blood – Validation Analysis Part 1 99
2.7.1.1 Objective 99
2.7.1.2 Validation Parameters 99
2.7.1.3 Acceptance Criteria 101
2.7.2 Surface Staining of CD3 CD4 CD28 in Whole Blood – Validation Analysis Part 2 101
2.7.2.1 Precision and Reproducibility 101
2.7.3 Measurement of soluble markers of inflammation – Validation Analysis 105
2.7.3.1 Objective 105
2.7.3.2 Precision and Reproducibility 105
2.8 Data Collection and Quality Control 107
2.9 Data Presentation and Statistical Analysis 108
2.9.1 Clinical Trial data presentation and statistical analysis 109

Chapter 3 The Phenotype of CD4+CD28null T-cells in AAV 112

3.1 Introduction 112
3.2 Cohort Characteristics 113
3.3 CD4+CD28null T-cell proportion and absolute count 115
3.3.1 AAV patients display a higher proportion of CD4+CD28null T-cells compared to HV 115
3.3.2 AAV patients have an inverted CD4:CD8 ratio compared to HV 117
3.4 CD4+CD28null T-cell phenotype and relationship to the CD4 CMV response 119

3.4.1 The size of CD4+CD28null T-cell expansions is closely linked to the overall CMV response in the CD4 compartment 121
3.4.2 Cytokine production by CD4+CD28null T-cells 125
3.4.3 Polyfunctionality of CD4+CD28null T-cells 128
3.4.4 Inhibitory receptor expression by CD4+CD28null T-cells 130
3.4.5 T helper phenotype 134
3.5 Discussion 139

Chapter 4 Association between CD4+CD28null T-cell expansions and cardiovascular disease risk in AAV 146

4.1 Introduction 146
4.2 CD4+CD28null T-cell expansions correlate with increased systolic blood pressure and pulse pressure 148
4.3 The CD4+CD28null T-cell percentage is independently associated with increased arterial stiffness 150
4.4 CD4+CD28null T-cell expression of endothelial receptors and markers of cytotoxicity 153
4.5 Discussion 158

Chapter 5 The potential for valaciclovir to prevent CMV mediated adverse modulation of the immune system in ANCA Associated VAScultis (CANVAS): a randomised controlled open label proof of concept clinical trial 163

5.1 Introduction 163
5.2 Recruitment and patient flow 164
5.3 Protocol compliance 166
5.4 Patient characteristics 168
5.5 Primary Outcome 171
5.6 Secondary Outcomes 177
5.6.1 Secondary Outcome – Change in the proportion of CD4+CD28null T-cells from baseline to 6 months 178

5.6.2 Secondary Outcome – Change in concentration of soluble markers of inflammation from baseline to 6 months 180
5.6.3 Safety 184
5.6.3.1 Adverse events 184
5.6.3.2 Transient rise in mean cell volume 187
5.6.3.3 Serious adverse events 188
5.7 Tertiary Outcome – Persistence of valaciclovir effect on the proportion of CD4+CD28null T-cells 189
5.8 Exploratory Outcomes 191
5.8.1 Change in CD4+CD28null T-cell counts from baseline to 6 months 191
5.8.2 Change in CD8CD28null T-cell percentage and absolute count from baseline to 6 months 192
5.8.3 Change in the CD4:CD8 ratio from baseline to 6 months 194
5.8.4 Change in CD4+CD28null CMV specific T-cells from baseline to 6 months..... 195
5.8.5 Change in soluble markers of endothelial damage from baseline to 6 months .. 197
5.8.6 Change in CMV IgG titre levels 200
5.8.6.1 Correlation between reduction in CMV IgG titre and CD4+CD28null T-cell parameters 203
5.8.7 Change in pulse wave velocity across the study period 204
5.9 CMV reactivation and expansion of the CD4+CD28null T-cell compartment in the control patients 206
5.10 CMV reactivation and polyfunctionality 210
5.11 Discussion 212

Chapter 6 General Discussion 219

6.1 Subclinical CMV reactivation drives the expansion of CD4+CD28null T-cells 220
6.2 Phenotype of CD4+CD28null T-cells in AAV and mechanisms of vascular damage 221
6.3 Control of subclinical CMV reactivation requires polyfunctional CD4 responses 223
6.4 Conclusion 225
6.5 Future work 226

References 228

Appendix 1 Publications and abstracts arising from this thesis at the time of submission 247
Appendix 2 Study Protocol 248
Appendix 3 Participant Information Sheet and Informed Consent Form 304
Appendix 4 Vicorder Work Instruction 311
Appendix 5 Data Input Quality Control Standard Operating Procedure 319
Appendix 6 CANVAS Laboratory Manual 324
Appendix 7 Validation Plans and Analysis for Clinical Trial Laboratory Assays 343
Appendix 8 Case Report Form 372
Appendix 9 Development Safety Update Report 379
Appendix 10 REC and MHRA approvals 391

List of Figures

Figure 1.1 Clinical features in AAV 5

Figure 1.2 AAV treatment algorithm 17

Figure 1.3 CD4 T-helper subsets 23

Figure 2.1 Study design diagram 53

Figure 2.2 SPIRIT figure showing study's schedule of enrolment, intervention and assessment. 61

Figure 2.3 Clinical Trial Sample Flow and Planned Analyses 71

Figure 2.4 Quantitation of CMV DNA in plasma and urine 77

Figure 2.5 CMV lysate titration 92

Figure 2.6 Flow cytometry data showing specificity of CMV lysate stimulation 93

Figure 2.7 Determination of CMV IgG titre 96

Figure 2.8 CMV IgG ELISA standard curve 98

Figure 2.9 CD3 CD4 CD28 staining validation 100

Figure 2.10 CD3 CD4 CD28 staining validation 103

Figure 3.1 CD4+CD28null and CD8CD28null T-cell expression in AAV and HV 116

Figure 3.2 CD4 count and CD4:CD8 ratio in AAV patients versus HV 118

Figure 3.3 CD4+CD28null T-cell responsiveness to CMV lysate 120

Figure 3.4 Correlation of CD4+CD28null T-cell proportion with the overall CD4 CMV response in AAV 124

Figure 3.5 Cytokine expression in CD4+CD28null T-cells – representative staining 126

Figure 3.6 Cytokine expression in CD4+CD28null T-cells in AAV and HV 127

Figure 3.7 Polyfunctionality of CMV responsive CD4+CD28null T-cells 129

Figure 3.8 Inhibitory receptor expression in CD4+CD28null T-cells – representative plots. 131

Figure 3.9 Inhibitory receptor expression in CD4+CD28null T-cells 132

Figure 3.10 Expression of inhibitory receptors and polyfunctionality 133

Figure 3.11 Expression of T-bet and BLIMP-1 – representative sample 134

Figure 3.12 Expression of T-bet and BLIMP-1 – summary results 136

Figure 3.13 T helper phenotype of CD4+CD28null T-cells 138

Figure 4.2 CD4+CD28null T-cells and pulse pressure 149

Figure 4.2 CD4+CD28null T-cells and pulse wave velocity 150

Figure 4.3 Expression of endothelial receptors and markers of cytotoxicity 155

Figure 4.4 NKG2D staining in CD4+CD28null T-cells 157

Figure 5.1 CONSORT flow diagram for the CANVAS clinical trial 166

Figure 5.2 Kaplan-Meier curve analysis of the primary outcome showing time to reactivation (>200 viral copies / mL; primary analysis) in the treatment (red line) and control (black line) groups 173

Figure 5.3 Kaplan-Meier curve analysis of the primary outcome showing time to reactivation (>20 viral copies / mL; secondary analysis) in the treatment (red line) and control (black line) groups 175

Figure 5.5 Secondary outcome – change in CD4+CD28null T-cell percentage during treatment period 179

Figure 5.6 Secondary outcome – change in plasma concentration of markers of inflammation 181

Figure 5.7 Secondary outcome – change in plasma concentration of markers of inflammation. Panels show absolute values for concentration of plasma markers at baseline (M0) and month 6 (M6). Panels A, C, E, G and I show values for TNF-α, IL-6, IL-10, IL-17 and CRP in the treated patients whilst panels B, D, F, H and J show absolute values for the concentration of the same markers at M0 and M6 for the control patients. 183

Figure 5.8 Transient macrocytosis in the treatment group 188

Figure 5.9 Change in CD4+CD28null T-cell proportion across the study period 190

Figure 5.10 Exploratory outcome – change in CD4+CD28null T-cell count during treatment period 192

Figure 5.11 Exploratory outcome – change in CD8CD28null T-cell proportion and absolute count during treatment period 193

Figure 5.12 Change in CD4:CD8 ratio during treatment period 194

Figure 5.13 Exploratory outcome – change in CMV specific CD4+CD28null T-cell proportion and absolute count during treatment period 196

Figure 5.14 Exploratory outcome – Change plasma concentration of markers of endothelial dysfunction (P-selectin)..... 198

Figure 5.16 Panels A and B show summary data of CMV IgG titre percentage change in treatment and control patients respectively 202

Figure 5.17 Correlation between CMV IgG titre and CD4+CD28null T-cell parameters 204

Figure 5.18 Change in pulse wave velocity across the study period..... 206

Figure 5.19 CMV reactivation and change in CD4+CD28null T-cells in controls 207

Figure 5.20 CD4+CD28null tracking experiments in control patients with CMV reactivation 209

Figure 5.21 Effect of polyfunctionality on risk of CMV reactivation 211

List of Tables

Table 1.1 2012 Chapel Hill Consensus Conference: definitions of anti-neutrophil cytoplasm antibody associated vasculitis [6] 3

Table 1.2 European Vasculitis Study Group Disease Severity Categories [58] 10

Table 2.1 Clinical Trial Endpoints 56

Table 2.2 Trial Management Group (TMG) 58

Table 2.3 Clinical Trial Inclusion and Exclusion Criteria 60

Table 2.4 Dose modification of valaciclovir according to creatinine clearance 66

Table 2.5 Antibodies used in the whole blood surface staining assay to determine CD4+CD28- proportions (secondary outcome) 80

Table 2.6 List of flow cytometry antibodies used for single stain compensation 81

Table 2.7 Antibodies used in a selection of whole blood surface staining experiments to determine T helper and memory sub-type 82

Table 2.8 Antibodies used in the whole blood surface staining assay to directly measure CD4 T-cells 82

Table 2.9 List of simplex analytes plexed for the determination of soluble markers of inflammation and endothelial damage in plasma 85

Table 2.10 Antibodies used to stain PBMC following overnight stimulation with CMV lysate or SEB 89

Table 2.11 Antibodies used to stain cells in CD4+CD28null T-cell tracking experiments 94

Table 2.12 Inter-assay variability and initial acceptance ranges for positive control used in the whole blood surface staining assay 105

Table 2.13 Coefficient of variation (CV) values for intra-assay and inter-assay variability for analytes used to inform the clinical trial secondary outcome 106

Table 3.1 Cohort characteristics for cross-sectional element of study 114

Table 3.2 Univariable analysis of factors associated with the size of the CD4+CD28null T-cell expansion in AAV patients 122

Table 3.3 Multivariable analysis of factors associated with the size of the CD4+CD28null T-cell expansion in AAV patients 124

Table 4.1 Factors associated with PWV on univariable analysis 151

Table 4.2 Multivariable model analysis of CD4+CD28null T-cell expansion and PWV 153

Table 5.1 Characteristics of patients randomised to the clinical trial 169

Table 5.2 Modifications to immunosuppression during clinical trial period 170

Table 5.3 CMV viral copy numbers per millilitre in clinical trial patients that reactivated CMV 172

Table 5.4 Contingency table showing number of reactivation episodes during the treatment period 177

Table 5.5 Results of paired ratio t tests for soluble markers of inflammation assayed for the clinical trial secondary outcome 182

Table 5.6 Adverse events reported during the clinical trial period 185

Table 5.7 Serious adverse events reported during the clinical trial period 189

Table 5.8 Repeated measures ANOVA analysis for CD4+CD28null T-cell percentage change across study period 191

Table 5.9 Results of paired ratio t tests for soluble markers of endothelial dysfunction 200

Abbreviations

AAV	ANCA associated vasculitis
ACS	Acute coronary syndrome
AE	Adverse event
ALT	Alanine transaminase
ANCA	Anti neutrophil cytoplasmic antibody
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATG	Anti thymocyte globulin
AZA	Azathioprine
BLIMP-1	PR domain zinc finger protein 1
BSA	Bovine serum albumin
CANVAS	CMV modulation of the immune system in ANCA associated vasculitis
CCR4	C-C motif chemokine receptor type 4
CCR5	C-C chemokine receptor type 5
CCR6	C-C motif chemokine receptor type 6
CCR7	C-C chemokine receptor type 7
CHCC	Chapel Hill Consensus Conference
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CRF	Clinical Research Facility
CRP	C reactive protein
CTCAE	Common Terminology Criteria for Adverse Events

CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CV	Coefficient of variation
CVD	Cardiovascular disease
CX3CR1	CX3C chemokine receptor 1
CXCR3	C-X-C motif chemokine receptor type 3
CYC	Cyclophosphamide
CYCLOPS	The Cyclophosphamide Daily Oral versus Pulsed Trial
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DSUR	Developmental safety update report
EBV	Epstein Barr virus
EDTA	Ethylenediamine tetra-acetic acid
eGFR	Estimated glomerular filtration rate
EGPA	Eosinophilic granulomatosis with polyangiitis
EM	Effector memory
EMRA	Revertant effector memory
ESRD	End stage renal disease
EUVAS	European vasculitis study group
FCS	Foetal calf serum
FHFF	Foetal human foreskin fibroblast
FMO	Fluorescence minus one
GC	Glucocorticoid
GCP	Good clinical practice
GI	Gastrointestinal
GPA	Granulomatosis with polyangiitis

HIV	Human immunodeficiency virus
HSP	Heat shock protein
HV	Healthy volunteer
ICAM-1	Intercellular Adhesion Molecule 1
ICOS	Inducible T-cell co-stimulator
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMPROVE	Mycophenolate Mofetil Versus Azathioprine for Maintenance Therapy in AAV
IP-10	Interferon gamma induced protein 10
IQR	Interquartile range
IRP	Immune risk profile
LAG-3	Lymphocyte activation gene 3
LFA-1	Lymphocyte Function Associated Antigen 1
MAP	Mean arterial pressure
MCP-1	Monocyte chemoattractant protein 1
MCV	Mean cell volume
MEPEX	Methylprednisolone Versus Plasma Exchange as Additional Therapy for Severe ANCA-Associated Glomerulonephritis
MHC	Major histocompatibility complex
MHRA	Medicines and healthcare products regulatory agency
MICA	Major histocompatibility complex class-I chain-related molecule A
MMF	Mycophenolate mofetil
MPA	Microscopic polyangiitis

MPO	Myeloperoxidase
MS	Multiple sclerosis
MTX	Methotrexate
NIHR	National Institute for Health Research
NK	Natural killer
NKG2D	Natural Killer Group 2 D
NORAM	Non-Renal Wegener's Alternatively Treated with Methotrexate
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PEXIVAS	Plasma Exchange and Glucocorticoid Dosing in the Treatment of AAV: an International Randomised Controlled Trial
PI	Principal investigator
PLEX	Plasma exchange
PR3	Proteinase 3
PSC	Primary sclerosing cholangitis
PWV	Pulse wave velocity
RA	Rheumatoid arhtirits
RANTES	Regulated on activation, normal T cell expressed and secreted
RAVE	Rituximab in ANCA-Associated Vasculitis
REC	Research ethics committee
RITAZAREM	Rituximab Vasculitis Maintenance Study
RITUXVAS	An International, Randomised, Open-label, Trial Comparing a Rituximab Based Regimen with a Standard Cyclophosphamide /

	Azathioprine Regimen in the Treatment of Active, “Generalised” AAV
RLV	Renal limited vasculitis
RTX	Rituximab
SAE	Serious adverse event
SEB	Staphylococcal enterotoxin B
SLE	Systemic lupus erythematosus
T-bet	T-box expressed in T-cells
TCR	T cell receptor
Th	T helper
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TMB	Tetramethyl benzidine
TMG	Trial management group
TNF-α	Tumour necrosis factor alpha
TSC	Trial steering committee
uACR	Urinary albumin creatinine ratio
UHB	University Hospital Birmingham
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen-4
WT	Wellcome Trust

CHAPTER 1

INTRODUCTION

Chapter 1 Introduction

1.1 ANCA associated vasculitis

1.1.1 Definitions and nomenclature

The ANCA associated vasculitides (AAV) are a group of systemic, autoimmune inflammatory conditions. They are primary small vessel vasculitides, and are characterised by the presence of circulating anti-neutrophil cytoplasm antibodies (ANCA) and a lack of immune complex deposition [1-3]. The AAV are typified by necrotising inflammation affecting small to medium blood vessels leading to end organ damage, commonly affecting the kidneys, lungs and upper airways [4] and include granulomatosis with polyangiitis (GPA; previously known as Wegener's granulomatosis), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA; previously known as Churg-Strauss) and renal limited vasculitis (RLV).

The AAV were officially defined according to the first International Chapel Hill Consensus Conference on the Nomenclature of Systemic Vasculitides (CHCC1994)[5] that was recently updated in 2012[6]. The CHCC 2012 definitions for the AAV are given in Table 1.1.

Table 1.1 2012 Chapel Hill Consensus Conference: definitions of anti-neutrophil cytoplasm antibody associated vasculitis [6]

ANCA-associated vasculitis (AAV)	Necrotising vasculitis, with few or no immune deposits, predominantly affecting small vessels (i.e. capillaries, venules, arterioles, and small arteries), associated with MPO ANCA or PR3 ANCA. Not all patients have ANCA. Add a prefix indicating ANCA reactivity, e.g., MPO-ANCA, PR3-ANCA, ANCA-negative
Granulomatosis with polyangiitis (GPA; Wegener's)	Necrotising granulomatous inflammation usually involving the upper and lower respiratory tract, and necrotising vasculitis affecting predominantly small to medium vessels (e.g., capillaries, venules, arterioles, arteries and veins). Necrotising glomerulonephritis is common.
Microscopic polyangiitis (MPA)	Necrotising vasculitis, with few or no immune depositis, predominantly affecting small vessels (i.e., capillaries, venules or arterioles). Necrotising arteritis involving small and medium arteries may be present. Necrotising glomerulonephritis is very common. Pulmonary capillaritis often occurs. Granulomatous inflammation is absent.
Eosinophilic granulomatosis with polyangiitis (EGPA; Churg Strauss)	Eosinophil-rich and necrotising granulomatous inflammation often involving the respiratory tract, and necrotising vasculitis predominantly affecting small to medium vessels, and associated with asthma and eosinophilia. ANCA is more frequent when glomerulonephritis is present.

ANCA: Anti-neutrophil cytoplasm antibody; MPO: Myeloperoxidase; PR3: Proteinase 3

1.1.2 Epidemiology of AAV

The annual incidence of AAV is approximately 20 per million with a point prevalence of 130/million for GPA and 47.9/million for MPA in the United Kingdom[1, 7, 8]. The peak age of onset is 65-74 with an incidence of 65 per million per year for that age group although the disease can occur at any age [8, 9]. AAV is more common in men however women tend to develop the disease at a younger age [10]. GPA is more common in the northern hemisphere and in particular within Caucasian populations of Northern Europe and the United States, whilst MPA is more common in Southern Europe, India and the Far East[7, 10-12]. EGPA is the least common of the AAV [12].

1.1.3 Clinical Features of AAV

Clinical features in AAV depend on the extent of organ involvement as well as the disease subtype. Many clinical features, particularly the non-specific inflammatory symptoms of malaise, fatigue, anorexia, weight loss, fever and joint and muscle aches often described as a 'flu-like' syndrome are common to all AAV [1, 4]. An array of other features exist (Figure 1.1) depending on the organs involved and the severity of the disease, with GPA more commonly affecting the ear, nose and throat and upper airways leading to symptoms such as epistaxis, nasal crusting, sinusitis, hearing loss and recurrent otitis media and MPA having a predilection for renal involvement frequently leading to rapidly progressing glomerulonephritis requiring renal replacement therapy [1, 4, 13-15]. Pulmonary manifestations range from focal

infiltrates and granulomas to life threatening pulmonary haemorrhage and haemoptysis, secondary to haemorrhagic alveolar capillaritis [4, 16].

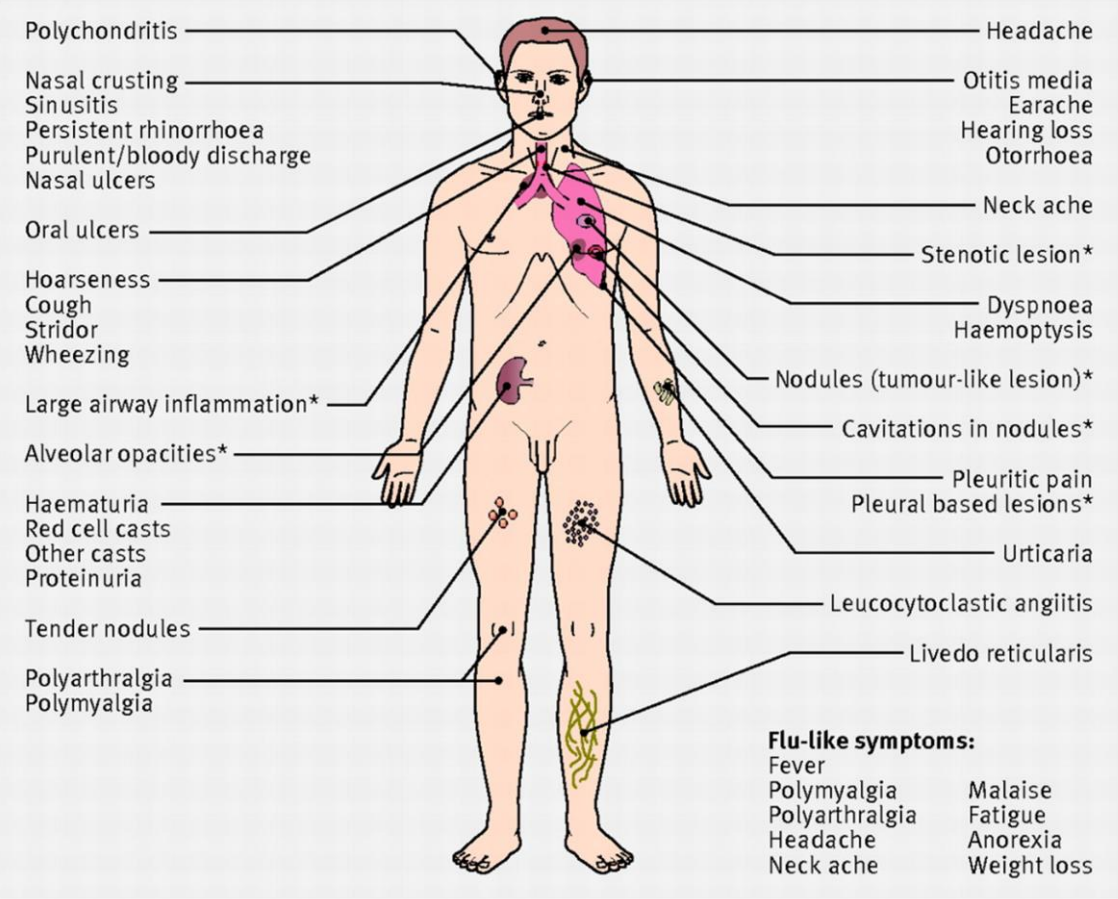

Figure 1.1 Clinical features in AAV

Constitutional flu-like symptoms are common to all AAV whilst other features depend on the extent of organ involvement and severity. * Indicate lesions that can be seen on chest radiography and computed tomography. Reproduced from [1] with permission from BMJ Publishing Group Ltd.

1.1.4 AAV pathophysiology

The last few decades have seen an abundance of studies investigating the pathophysiology of AAV. Although EGPA is included in the umbrella term of AAV, its pathophysiology is significantly different from that of GPA and MPA and will not be discussed here.

ANCA have been associated with AAV since their first description in 1985 [17]. ANCA are categorised as cytoplasmic (c-ANCA) or perinuclear (p-ANCA) depending on their staining pattern by indirect immunofluorescence. The main antigenic targets of ANCA are proteinase 3 (PR3) and myeloperoxidase (MPO), located in the azurophilic granules of neutrophils and the peroxidase positive lysosomes of monocytes [18]. PR3 specific ANCA usually exhibit a cytoplasmic pattern and are preferentially seen in GPA whilst MPO specificity tends to be associated with a perinuclear pattern and MPA or RLV [19]. Other antigen specificities such as human lysosomal membrane protein 2 have also been recently described [20, 21].

Initial evidence for the pathogenicity of ANCA came from *in vitro* studies where both PR3 and MPO ANCA were shown to be able to activate neutrophil activation leading to oxidative burst and degranulation. This effect was dose dependent and was significantly enhanced following neutrophil priming with TNF-α [22]. In vivo evidence for MPO-ANCA pathogenicity was provided by mouse experiments performed by Xiao

and colleagues where the transfer of murine MPO-ANCA to wild-type or recombinant activating gene 2-deficient ($Rag2^{-/-}$) mice led to glomerulonephritis suggesting that even in the absence of T-cells and B-cells MPO-ANCA alone are sufficient to cause disease [23]. Studies utilising the MPO ANCA mouse model have shown that disease induction can be ameliorated via several ways including neutrophil depletion, inhibition of plasma cell function and blocking of proinflammatory cytokines such as TNF-α. Recently, complement has also been implicated in AAV pathogenicity with experiments showing that blocking the complement component C5 can ameliorate the disease process [23-30]. On the other hand, there is no convincing animal model to date that is able to replicate the granulomatous inflammation seen in PR3-ANCA mediated disease, suggesting that cellular effector mechanisms play a significant role in the development of GPA.

In summary, our current model for tissue injury in AAV involves the binding of ANCA on surface expressed MPO and PR3 auto-antigens on circulating cytokine-primed neutrophils, resulting in neutrophil adhesion and transmigration on activated endothelial cells with ensuing neutrophil degranulation and endothelial tissue injury ultimately leading to necrotising vasculitis [31, 32].

1.1.4.1 Involvement of T-cell immunity in AAV pathophysiology

As alluded to earlier, T-cells have also been shown to be implicated in AAV pathophysiology. ANCA are class switched IgG antibodies, implying a requirement for T-cell help as well as a breach in self-tolerance giving rise to auto-reactive T-cells [33]. Furthermore, a reduction in the proportion and functionality of regulatory T-cells has previously been found in AAV patients [34] whilst multiple studies have shown evidence of persistent activation of T-cells in patients with MPA and GPA as well as infiltrating T-cells in granulomatous lesions [35-38]. Finally, T-cell depleting agents such as anti-thymocyte globulin and the anti-CD52 antibody alemtuzumab can induce remission in refractory cases of AAV suggesting a significant role for T-cell mediated immunity in disease pathophysiology [39, 40].

Indeed, multiple lines of evidence have recently suggested involvement of T-cells in AAV pathophysiology. T-helper cells (see also section 1.2) are aberrantly polarised in AAV with a predominance of a Th1 pattern seen in localised GPA and MPA and a mixture of Th1 and Th2 CD4-cell involvement in active generalised GPA [41-44]. IL-17 secreting CD4 T-cells (Th17) have recently been shown to be a major pathogenic cell subset involved in the trigger of autoimmunity and the establishment of inflammation [45-47]. IL-17 promotes the release of proinflammatory cytokines essential for triggering the surface expression of MPO and PR3 on activated neutrophils and auto-antigen specific Th17 cells are relatively increased in ANCA positive patients in comparison to ANCA negative patients and controls [46-51].

Persistent T-cell activation has been observed in AAV and has been found to correlate with disease activity and severity [52]. Furthermore an expansion of a subset of CD4 T-cells, known as effector memory CD4 T-cells (EM), has been described in AAV patients together with a reciprocal decrease in naïve CD4 T-cells [37]. Effector memory T-cells are found in increased numbers in AAV patients in remission compared to controls. Interestingly their proportion in peripheral blood has been shown to decline during active renal disease in patients with AAV in parallel with an increase in the presence of CD4 EM T-cells in the urinary sediment, suggesting migration towards target organs during episodes of disease activity [53].

Finally, CD4 T-cells lacking the co-stimulatory molecule CD28 that comprise a cytotoxic T_{EM} subset (CD4+CD28null T-cells; discussed in detail in Section 1.2) are also expanded in AAV patients [54]. These CD4+CD28null T-cells have been found in abundance in the granulomatous lesions of patients with GPA [35, 36]. They possess cytotoxic abilities reminiscent of natural killer cells and have been shown to express the activating C-type lectin-like homodimeric receptor NKG2D [55]. The ligand for NKG2D, major histocompatibility complex class-I chain-related molecule A (MICA), is in turn upregulated in glomerular epithelial cells in active renal AAV disease and is expressed in granulomatous lesions of GPA patients [56, 57].

1.1.5 Treatment of AAV

The principles of treatment in AAV are to induce and maintain remission utilising the minimum necessary immunosuppression that is appropriate to disease severity. Induction of remission is achieved by the use of highly potent immunosuppressive agents that generally target T-cells or B-cells. The European Vasculitis Study Group (EUVAS) provided a framework of disease severity in order to guide the development of clinical trials in the treatment of AAV and harmonise approaches to treatment (Table 1.2) [58].

Table 1.2 European Vasculitis Study Group Disease Severity Categories [58]

Localised	Upper and / or lower respiratory tract disease without any other systemic involvement or constitutional symptoms.
Early systemic	Any system, without organ-threatening or life threatening disease.
Generalised	Renal or other organ-threatening disease, serum creatinine < 500 μmol/L.
Severe	Renal or other vital organ failure, serum creatinine > 500 μmol/L.
Refractory	Progressive disease unresponsive to glucocorticoids and cyclophosphamide.

The majority of AAV clinical trials carried out to date have been based on the EUVAS classification of severity. A brief account of some important studies that have shaped current treatment strategies in AAV follows.

1.1.5.1 Induction of remission in AAV

In early systemic disease, comparison of induction therapy with oral corticosteroids and either cyclophosphamide or methotrexate (Non-Renal Wegener's Alternatively Treated with Methotrexate; NORAM) demonstrated methotrexate to be as effective as cyclophosphamide in inducing remission [59] although long-term follow up revealed that patients treated with methotrexate relapsed more quickly and were more likely to receive further immunosuppression [60].

In generalised disease, use of pulsed cyclophosphamide therapy as opposed to daily oral cyclophosphamide in addition to oral corticosteroids was shown to be equivalent in terms of achieving remission (The Cyclophosphamide Daily Oral versus Pulsed (CYCLOPS) Trial) [61]. Patients in the pulsed cyclophosphamide arm received a lower cumulative dose of cyclophosphamide and had fewer episodes of leucopoenia; however long-term follow up revealed a higher risk of relapse in the pulsed group, although rates of renal failure and mortality were equivalent [62].

Two recent trials considered the monoclonal anti-CD20 antibody rituximab as an alternative to cyclophosphamide for induction of disease remission in patients with generalised or severe AAV. RITUXVAS (An International, Randomised, Open-label, Trial Comparing a Rituximab Based Regimen with a Standard Cyclophosphamide / Azathioprine Regimen in the Treatment of Active, "Generalised" AAV) and RAVE (Rituximab in ANCA-Associated Vasculitis) had important differences in their methodology, however both trials concluded that rituximab was at least as effective as cyclophosphamide in achieving remission whilst the rate of adverse events including infection were comparable between rituximab and cyclophosphamide treated patients [63, 64].

Given that *in vitro* studies have revealed a role for tumour necrosis factor-alpha (TNF-α) in activating neutrophils in AAV pathophysiology [24], anti-TNF monoclonal antibodies such as infliximab, adalimumab and etanercept have also been utilised for induction of remission in AAV [65-68]. The addition of infliximab to standard therapy with corticosteroids and cyclophosphamide was shown not to lead to additional benefit [67] whilst adalimumab and etanercept demonstrated some improvement in disease activity scores and some limited evidence of reduced corticosteroid exposure [66, 68]. Due to the open-label, non-randomised nature of these studies as well as their mixed results, TNF blockade is reserved for cases of refractory disease where patients have not responded to conventional therapy.

In the management of severe life-threatening disease, the Methylprednisolone Versus Plasma Exchange as Additional Therapy for Severe ANCA-Associated Glomerulonephritis (MEPEX) trial, randomised patients with serum creatinine > 500 μmol/L to receive either three 1 g infusions of methylprednisolone on consecutive days or seven 60 mL/kg treatments of plasma exchange within 14 days. All patients also received high dose oral corticosteroids and daily oral cyclophosphamide for induction of remission followed by azathioprine for maintenance of remission [69]. Patients randomised to the plasma exchange arm exhibited better renal recovery and this difference was maintained at 12 months although subsequent long-term follow up has shown no difference between the two arms in terms of survival or renal function at a median follow-up of 3.95 years [70].

The Plasma Exchange and Glucocorticoid Dosing in the Treatment of AAV: an International Randomised Controlled Trial (PEXIVAS; ClinicalTrials.gov Identifier: NCT00987389) is an ongoing multi-centre trial that aims to further clarify the efficacy of plasma exchange in improving renal recovery in patients with glomerulonephritis secondary to AAV as well as determine whether a reduced corticosteroid dose is associated with similar efficacy to the conventional dose but an improved adverse even rate.

A recent retrospective study showed that pulsed cyclophosphamide in addition to high dose corticosteroids and plasma exchange was associated with a favourable outcome

when compared to the MEPEX arm that received daily oral cyclophosphamide suggesting that pulsed cyclophosphamide is a viable alternative also in severe disease to reduce cumulative dose of cyclophosphamide and ensuing toxicity [71].

1.1.5.2 Maintenance of remission in AAV

AAV is associated with a high rate of relapse that is seen in 50% of patients within the first 5 years following diagnosis and commencement of therapy [72-75]. Long-term maintenance immunosuppression is therefore usually necessary to adequately suppress disease activity. Choice of maintenance immunosuppression must be balanced with the toxicity of available agents. Cyclophosphamide was initially used to induce as well as maintain remission for prolonged periods of time resulting in high rates of infection and malignancy, particularly urothelial cancer [76, 77]. The Cyclophosphamide Versus Azathioprine for Early Remission Phase of Vasculitis (CYCAZAREM) trial showed that switching to oral azathioprine for maintenance of remission after 3-6 months of oral cyclophosphamide treatment was associated with equal rates of relapse and adverse events compared to continuing oral cyclophosphamide until month 12 [78].

The effectiveness of mycophenolate mofetil as a remission maintenance agent was compared to azathioprine in the Mycophenolate Mofetil Versus Azathioprine for Maintenance Therapy in AAV (IMPROVE) trial that demonstrated mycophenolate

mofetil to be less effective than azathioprine with a significantly higher rate of relapse [79]. The preferred agent for maintaining remission is therefore azathioprine although mycophenolate mofetil remains an option for patients that are intolerant to azathioprine. Finally, rituximab has been described as an effective agent to maintain remission in several large case series [80-84] and a clinical trial (Rituximab Vasculitis Maintenance Study; RITAZAREM; ClinicalTrials.gov Identifier: NCT01697267) is currently underway to evaluate the effectiveness of rituximab in maintaining remission compared to azathioprine.

1.1.5.3 Treatment of refractory AAV disease

Refractory disease that is unresponsive to conventional therapy remains a very difficult area in the treatment of AAV. To date there has only been one randomised controlled trial that randomly assigned patients to receive either intravenous immunoglobulin (IVIG) or placebo infusions for 5 days and showed short term reduction in disease activity in the IVIG treated group [85]. Various agents such as rabbit anti-human thymocyte globulin (ATG) [40], the lymphocyte depleting antibody alemtuzumab (anti-CD52) [86], the immunosuppressant 15-deoxyspergualin [87-89], infliximab [90] and rituximab [91] have been utilised in the treatment of refractory or frequently relapsing AAV disease with mixed results although a detailed discussion of these studies is beyond the scope of this thesis.

1.1.5.4 Current treatment recommendations for AAV

Recently published guidelines by the British Society of Rheumatology / British Health Professionals in Rheumatology have suggested that all patients with AAV should be considered to have severe, potentially life-threatening or organ-threatening disease [92]. The proposed treatment algorithm (Figure 1.2) therefore suggests consideration of 'stepping down' induction therapy to methotrexate or mycophenolate mofetil in addition to corticosteroids instead of cyclophosphamide or rituximab and corticosteroids in cases of no organ threatening involvement and increasing the intensity of induction therapy by adding plasma exchange to high dose corticosteroids and cyclophosphamide or rituximab in the presence of vital organ or life-threatening involvement [92].

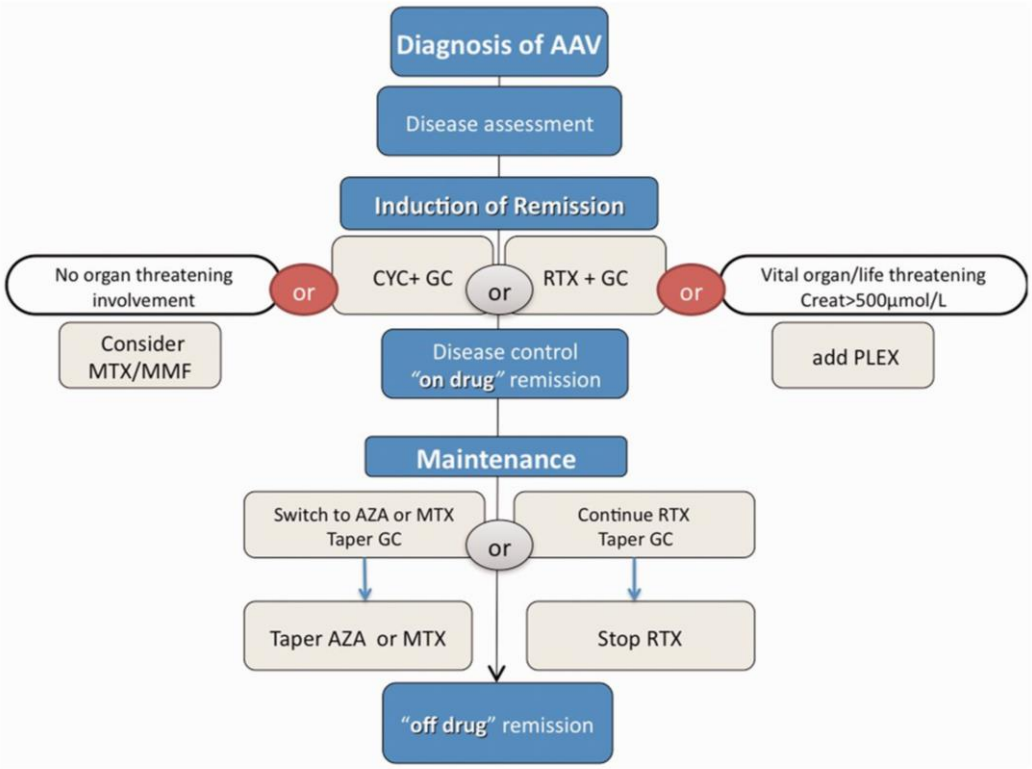

Figure 1.2 AAV treatment algorithm

Following disease assessment, induction of remission is achieved by the use of glucocorticoids (GC) and cyclophosphamide (CYC) or rituximab (RTX). Methotrexate (MTX) or mycophenolate mofetil (MMF) can be considered in place of CYC or RTX in cases of no organ threatening involvement whilst plasma exchange (PLEX) should be added in cases of vital organ / life threatening features. Maintenance is with azathioprine (AZA), MTX or RTX and gradual tapering of GC. Reproduced from [92] with permission from Oxford University Press on behalf of the British Society for Rheumatology.

1.1.6 Morbidity and mortality in AAV

1.1.6.1 Prognosis

Prior to the use of treatment regimens that employ cyclophosphamide and high dose corticosteroids, AAV was almost universally fatal with 2-year survival rates of less than 20% [74]. Current 5-year survival for AAV is approximately 80% [93] and although the disease remains life-threatening and potentially fatal if left untreated, modern therapeutic strategies as described in the previous section have transformed AAV into a chronic inflammatory condition with a relapsing-remitting course.

1.1.6.2 Infection and cardiovascular disease in AAV

Nevertheless, following induction of remission, AAV patients have a mortality ratio of 2.6 compared to an age and gender matched general population [93]. Furthermore, although survival has greatly improved, one of the most significant challenges facing clinicians treating AAV patients at present is reducing treatment associated morbidity and mortality, particularly related to infection. Indeed, a recent study that examined long-term prognosis in 535 patients with AAV that participated in four EUVAS clinical trials between 1995 and 2002 found that active disease was the cause of death in 19% of cases whereas infection accounted for 48% of deaths amongst patients who died within the first year after enrolment. Following the first year, patients died mainly due to cardiovascular disease (26%), malignancy (22%) and again infection (20%). As such,

infection and cardiovascular disease (CVD) represent the two most importance sources of morbidity and mortality in AAV.

A matched pair cohort study has further highlighted the importance of CVD as a significant cause of morbidity and mortality in AAV [94]. In this study patients with AAV had a significantly higher incidence of cardiovascular events, with a hazard ratio of 2.23, compared to matched patients with non-inflammatory chronic kidney disease (CKD), a group of patients already at an increased risk of CVD compared to the general population [95-97].

Other studies have shown that patients with AAV display enhanced atherosclerosis [98] with increased surrogate markers of CVD such as endothelial dysfunction [99-101], arterial stiffness [102], carotid artery intima-media thickness [103] and a high frequency of arterial plaques [104], whilst further observational studies have observed increased morbidity from ischaemic heart disease in patients with AAV [98, 105]. The inflammatory nature of atherosclerosis, the underlying lesion in CVD, is becoming increasingly appreciated and excess mortality associated with other systemic inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus has been attributed to accelerated atherogenesis leading to CVD [106-111].

Some of the clinical trials and studies discussed in the previous section have aimed to address the challenge of treatment related morbidity, particularly in relation to infection, for example by utilising pulsed cyclophosphamide rather than continuous daily cyclophosphamide [61, 71] in an effort to reduce overall dosage and toxicity, whilst other ongoing trials such as PEXIVAS aim to address whether it is possible to use lower doses of corticosteroids.

Nevertheless there exists a significant unmet need to reduce the burden of infection and cardiovascular disease, two of the leading causes of death in patients with AAV.

1.1.6.3 Dysregulated T-cell immunity contributes to AAV morbidity

Aberrant T-cell immunity is relevant in the pathophysiology of disease related morbidity in AAV. Indeed, Morgan *et al.* showed that in patients with AAV the size of the effector memory CD4+CD28null T-cell subset (discussed in detail in the next section) was correlated with an increased risk of infection, reduced renal function and increased mortality [112]. Importantly, expansion of CD4+CD28null T-cells in that study was not confined to AAV patients as both patients and controls exhibited similar proportions of CD4+CD28null T-cells. CD4+CD28null T-cells however, were specific for Cytomegalovirus (CMV) seropositivity as only CMV seropositive AAV patients and CMV seropositive healthy controls exhibited significant expansions of CD4+CD28null T-cells. As such, dysregulation of T-cell immunity in AAV significantly impacts prognosis.

1.2 CD4+CD28null T-cells

1.2.1 Organisation of the immune system

The human immune system consists of an innate system that constitutes the first line of defence against foreign invading pathogens [113] and an acquired adaptive part that develops in response to infection, is able to recognise small differences in foreign molecules, recognise and eliminate foreign pathogens as well as develop immunological memory in order to provide an effective response upon re-challenge [114]. The lymphocyte population of the immune system is predominantly comprised of natural killer cells (NK) of the innate system and T-cells and B-cells that form part of the adaptive system. In addition, other less common cell types such as NK T cells and $\gamma\delta$ T cells have been described [115-117].

T-cells derive their prefix T from the fact that they mature in the thymus as opposed to B-cells that mature in the bone marrow [118]. T-cells are involved in cellular immunity where foreign antigens are recognised and suppressed by a cellular immune response, as well as in the generation and maintenance of self-tolerance. Three main types of T-cells have been characterised to date, cytotoxic T-cells (T_C), helper T-cells (T_H) and regulatory T-cells (T_{REG}). Cytotoxic and helper T-cells can be distinguished by the presence of CD8 and CD4 membrane glycoproteins on their surface, with CD4 T-cells generally functioning as helper T-cells [119]. The ratio of CD4+ to CD8+ T-cells is normally approximately 2:1 although this can be altered in various disease states [120, 121] as discussed further in Section 1.3.4.

1.2.2 CD4 T-cells and their subsets

Following activation CD4 T-helper cells, the key regulators of the immune system, are able to differentiate into various T_H cell lineages with distinct biological functions (Figure 1.3).

1.2.2.1 T-cell activation

The traditional model of T-cell activation requires two signals for optimal activation of CD4 T-cells. The first signal is provided by the interaction of the antigen with the major histocompatibility complex (MHC) and the T-cell receptor (TCR) whilst the second signal involves co-stimulatory cell surface molecule interaction on the antigen presenting cell (APC) with stimulatory receptors on the surface of the CD4 T-cell [122] (Figure 1.3). The best characterised co-stimulatory pathway is the CD80/CD86 – CD28/CTLA-4 pathway where CD80 and CD86 represent ligands on the APC and CD28 and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) represent stimulatory and inhibitory receptors on the surface of CD4 T-cells that orchestrate the responses of naïve, memory and effector cells [122-125]. In the absence of appropriate co-stimulatory signals T-cells are thought to enter a state of anergy where proliferation and effector functions can be inhibited [126]. However, recently other alternative pathways of T-cell stimulation and inhibition including new members of the B7-CD28 superfamily such as ICOS (inducible T-cell co-stimulator) and PD-1 (programmed death 1) have been described [127, 128] and it is now accepted that other receptor ligand

interactions can support T cell proliferation and development of effector functions [129-131].

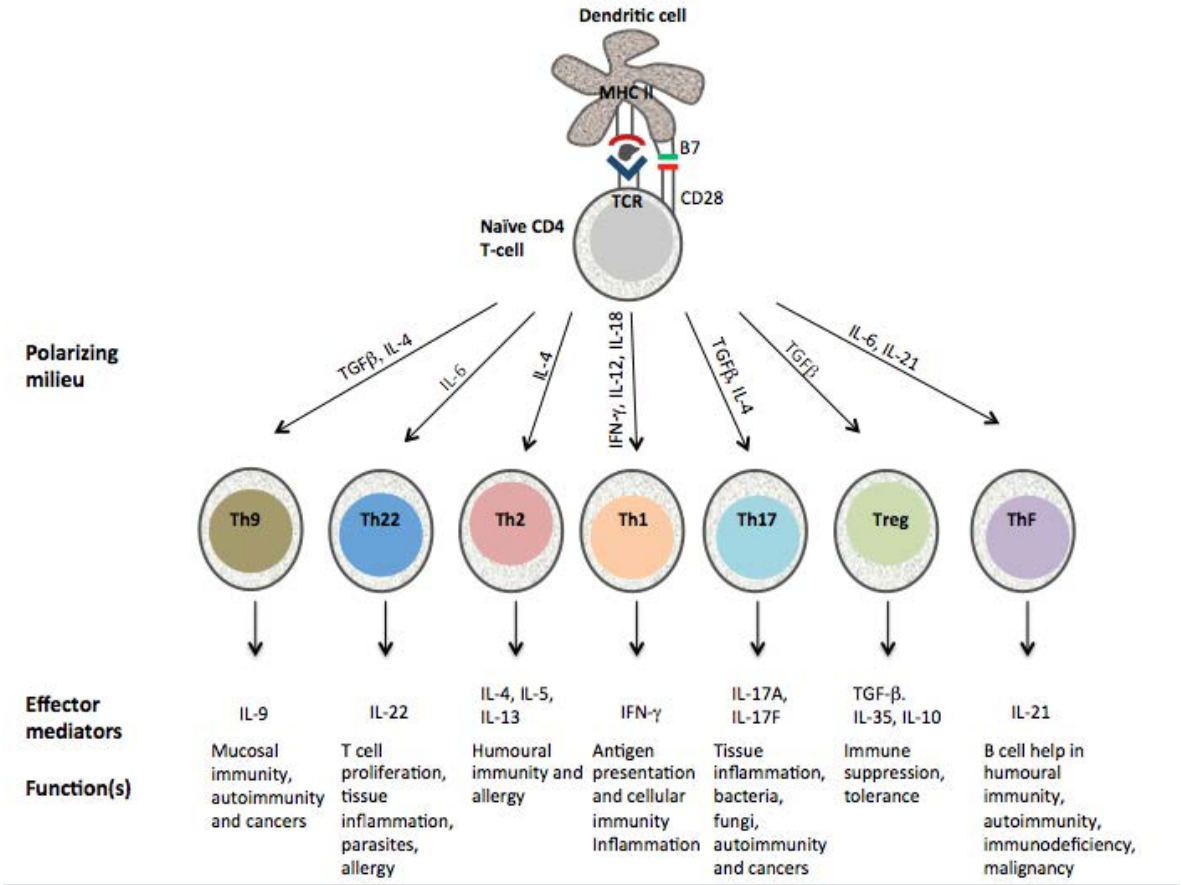

Figure 1.3 CD4 T-helper subsets

Following interaction with antigen presenting cells and depending on the nature of antigen stimulation signal received naïve CD4 T-cells differentiate into distinct T-cell subsets. This process is enabled by recognised cytokines that provide the relevant 'polarising milieu' and different CD4 T-cell subsets in turn are associated with secretion of key cytokines and distinct functions.

Treg = T regulatory; ThF = T follicular

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1.2.2.2 CD4 T-cell memory differentiation

As alluded to CD4 T-cells can differentiate into distinct subtypes (Figure 1.3). For some of these subtypes unique patterns of chemokine receptor expression have been described as a way of identification. For example Th1 cells have recently been described to express the chemokine receptor CXCR3 (C-X-C motif chemokine receptor type 3) but lack expression of CCR6 (C-C motif chemokine receptor type 6) and CCR4 (C-C motif chemokine receptor type 4) whereas Th2 cells express CCR4 but lack CXCR3 and CCR6 expression whilst Th17 cells express both CCR4 and CCR6 but not CXCR3 [133].

Th1 cells produce the type 1 cytokines interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin 2 (IL-2) and are crucial in activating macrophages and enabling cell mediated immunity against intracellular pathogens [134]. Recently the role of Th1 cells in the development and exacerbation of inflammation in autoimmune disease as already discussed as well as in vascular pathology is increasingly being recognised [135, 136].

CD4 T-cells can also phenotypically be described with regards to their differentiation status from naïve to effector and memory lymphocytes. Naïve T-cells express the CD45RA isoform of CD45 as opposed to memory T-cells that express the CD45RO isoform and are generally negative for CD45RA expression [137]. In addition, naïve T-

cells express the cell adhesion molecule L-selectin (CD62L) and the chemokine receptor CCR7 (C-C chemokine receptor type 7) and are able to circulate between peripheral blood and lymphoid tissue [138]. Following activation, naïve T-cells differentiate into effector cells. Following clearance of the antigen, a large contraction phase ensues with some survivors differentiating to become memory cells that can confer long term protection and give rise to an enhanced response upon secondary antigenic challenge [139, 140].

Memory T-cells are CD45RA negative and can be further sub-divided into central (CM) and effector memory (EM) subsets. Central memory T-cells maintain expression of CD62L and CCR7 and are able to migrate into lymph nodes to mount secondary proliferative responses but lack effector functions [141]. Effector memory T-cells on the other hand exhibit heterogeneous expression of CD62L and have lost expression of CCR7. They express characteristic sets of chemokine receptors and adhesion molecules, home into inflamed tissues and display immediate effector function [141, 142]. Effector memory T-cells were originally described within the CD8 compartment but recently the existence of CD4 EM T-cells is increasingly being recognised [143, 144]. As EM T-cells differentiate further they lose expression of CD27 and CD28 and gain expression of CD57 with a concomitant increase in cytolytic activity reflected by the ability to produce cytotoxic molecules like perforin, granzyme A and granzyme B [145]. Revertant EM T-cells are highly differentiated cells that have re-expressed CD45RA (EMRA) and show features of senescence and terminal differentiation [142].

1.2.3 CD28 expression and downregulation

CD28 is constitutively expressed on the surface of CD4 and CD8 T-cells [122]. It is essential for optimal activation of T-cells and deletion of the CD28 gene in mice results in severe immunocompromise with reduced T-helper activity and impaired production of immunoglobulins [146], although the generation of T-cell cytotoxicity in these experiments was not affected suggesting the presence of alternate co-stimulatory pathways that can induce cytokine production [129, 146]. CD28 expression can be down-regulated leading to CD4 and CD8 T-cells that lack expression of CD28 (CD4+CD28null; CD8+CD28null) that are now commonly seen with increasing age in healthy individuals as well as in the presence of chronic infection [147-151]. CD4+CD28null T-cells lack CD28 mRNA indicating that CD28 downregulation occurs via transcriptional silencing that is reversible with IL-12 administration [147, 152].

1.2.4 Characteristics of CD4+CD28null T-cells

CD4+CD28null T-cells were first described in patients with rheumatoid arthritis (RA) [153, 154] but have since then been described in association with a multitude of inflammatory disorders [54, 155-167], viral infections [150, 168-170], cardiovascular disease [171-181] and in the context of an ageing immune system where they have been found to represent as much as 50% of the total CD4 compartment [149, 151, 182].

They exhibit important differences compared to their CD4+CD28+ counterparts in that they are oligoclonal with limited TCR diversity [153, 154, 183], the loss of CD28 and oligoclonality both suggestive of repeated exposure to a persistent antigen [184]. Furthermore, they show evidence of an increased replicative history as they have been found to contain significantly shortened telomeres [185, 186]. Despite that, CD4+CD28null T-cells are relatively resistant to apoptosis and are long lived [129, 182, 185, 187]. They exhibit cytotoxic properties with the ability to produce Th1 type cytokines IFN-γ and TNF-α as well as cytolytic enzymes such as granzyme B and perforin [167, 188-191]. They express the chemokine receptor CXCR3 and in that respect, resemble the chemokine receptor expression pattern of Th1 cells suggesting that they can be classified as cytotoxic Th1 type cells [184, 192].

In addition, CD4+CD28null T-cells have been shown to express molecules that are usually found on the surface of cells of the innate immune system as well as an array of surface adhesion molecules. CD4+CD28null T-cells express receptor molecules typically found on NK cells such as CD57 and CD11b [184], the latter being important in regulating leukocyte endothelial adhesion and inflammation [193], although they do not express the NK specific molecule CD16. Furthermore, they do not express the invariant TCR α-chain and therefore cannot be characterised as NK T cells [194]. NKG2D receptors have been shown to be expressed by CD4+CD28null T-cells in RA, giant cell arteritis, polymyalgia rheumatica and AAV [55, 195-197] while other killer cell immunoregulatory inhibitory receptors (KIR) such as KIR2DS2 have been found on

CD4+CD28null T-cells from acute coronary syndrome (ACS), chronic kidney disease and RA patients [196, 198-200]. In RA the presence of KIR2DS2 is considered a risk factor for the development of vasculitis but not synovitis [184].

As mentioned earlier, CD4+CD28null T-cells express CXCR3, the ligand of which, IP-10 (interferon gamma-induced protein 10), has been implicated together with CXCR3 in the pathophysiology of inflammatory arthritis and atherosclerosis [201]. However, differential expression of CXCR3 has been observed in certain patient groups with psoriasis patients exhibiting a decreased proportion of CXCR3 expressing CD4+CD28null T-cells in the periphery [202]. Similarly, although CD4+CD28null T-cells from RA patients have been found to express CCR5 (C-C chemokine receptor type 5) thereby being able to traffic to the inflamed synovium [203], CCR5 expression has been shown to be very low in CD4+CD28null T-cells of ankylosing spondylitis patients [167] suggesting differential expression of chemokine receptors by CD4+CD28null T-cells in different inflammatory conditions. Patients with RA and multiple sclerosis (MS) have also been shown to express the VLA-4 (Very Late Antigen-4) and LFA-1 (Lymphocyte Function Associated Antigen 1) receptors whose ligands VCAM-1 (Vascular Cell Adhesion Molecule-1) and ICAM-1 (Intercellular Adhesion Molecule 1) are found on endothelial cells as well as in soluble form and on APC (ICAM-1) [184].

Another important chemokine receptor expressed by CD4+CD28null T-cells is the receptor for fractalkine, CX3CR1 (CX3C chemokine receptor 1). Fractalkine, or CX3CL1,

exists in a soluble as well as a membrane bound form and functions as both a chemoattractant (soluble form) and an adhesion molecule (membrane bound form) promoting shear resistant adhesion of CX3CR1 expressing leukocytes [204]. The role of fractalkine is increasingly being recognised in inflammatory pathology including atherosclerosis and renal disease [205-207] and membrane bound fractalkine can be induced on endothelial cells via activation by Th1 type inflammatory cytokines such as IFN-γ and TNF-α [208]. CX3CR1 is expressed on CD4+CD28null T-cells from RA and MS patients. In RA, activated synoviocytes express fractalkine [209-211], whereas in MS the chemokine gradient of soluble fractalkine induces migration of CD4+CD28null T-cells in inflamed brain lesions [163].

1.2.5 CD4+CD28null T-cells are associated with inflammation

Given the characteristics of CD4+CD28null T-cells discussed above these cells are thought to represent a proinflammatory cytotoxic subset. Furthermore, it has been shown that an inflammatory milieu contributes to the expansion of CD4+CD28null T-cells explaining the association between this cell subset and chronic inflammatory conditions as well as with advancing age owing to the increase in proinflammatory cytokines that is known to occur with age [212-214]. In RA, Bryl *et al.* have shown that TNF-α induces downregulation of surface expressed CD28 through transcriptional repression by inhibiting the binding of nuclear protein complexes that recognise regulatory sequences [212]. On the other hand, treatment with the anti-TNF-α

monoclonal antibody infliximab in RA as well as in unstable angina patients leads to partial reappearance of the CD28 molecule on the CD4+ cell surface [175, 215-217].

1.2.5.1 CD4+CD28null T-cell expansion in inflammatory disease states

Following the original description of CD4+CD28null T-cells in RA [153, 154], expansions of these cells have been reported in a multitude of inflammatory conditions. Recently they have been implicated in the pathogenesis of primary sclerosing cholangitis (PSC) where CX3CR1 expressing CD4+CD28null T-cells were found in higher frequencies in liver tissue from PSC patients compared to primary biliary cirrhosis or non-alcoholic steatohepatitis [218]. In the same study, TNF-α was found in abundance in PSC liver tissue and TNF-α downregulated the expression of CD28 *in vitro*, an effect that was prevented by administration of 1,25(OH)2D3 vitamin D. CD4+CD28null T-cells have been shown to induce muscle cell death *in vitro* via secretion of granzyme B and perforin in experiments utilising cells from patients with polymyositis [219].

CD28 downregulation on CD4 T-cells is associated with poor prognosis in patients with pulmonary fibrosis [220], extra-articular disease such as rheumatoid nodules and vasculitis in RA [221] and *in vitro* experiments have shown that CD4+CD28null T-cells may be implicated in the pathophysiology of MS, an effect that is augmented by IL-15 [162, 163]. The frequencies of CD4+CD28null T-cells in RA and healthy individuals follow a bimodal distribution, defining carriers and non-carriers [221]. Given the

extensive association with inflammatory and autoimmune disease it has been postulated that CD4+CD28null T-cells are autoreactive in nature. However, studies have failed to show auto-reactivity of these cells to RA or MS autoantigens and thus CD4+CD28null T-cells are more likely to cause pathology via indirect ways [159].

1.2.5.2 CD4+CD28null T-cells in AAV

Expansions of CD4+CD28null T-cells have been repeatedly observed in the peripheral blood of AAV patients and have been found in abundance in bronchoalveolar lavage fluid and within granulomas of patients with GPA [35, 36, 54, 222, 223]. The degree of expansion of CD4+CD28null T-cells has been shown to be proportional to the cumulative number of involved organs and hence disease severity across time but not with severity of acute disease or treatment [54]. These findings were confirmed in a recent study that showed no difference in the proportion of CD4+CD28null T-cells between AAV patients with active disease versus AAV patients in remission [224]. On the other hand, as already alluded to earlier, CD4+CD28null T-cell expansions in AAV patients have been associated with reduced kidney function, increased risk of infection and increased mortality [112].

1.2.5.3 CD4+CD28null T-cells in renal disease

Expansions of CD4+CD28null T-cells have also been described in patients with other immune mediated renal disease, chronic kidney disease (CKD) and end-stage renal

disease (ESRD). In systemic lupus erythematosus (SLE), CD4+CD28null T-cells have been found to be independently associated with disease damage [155] echoing the findings in AAV.

Yadav et al. found that amongst ESRD dialysis patients CD4+CD28null T-cells were cytotoxic as judged by their ability to produce granzyme B and perforin and were significantly expanded compared to healthy volunteers. Furthermore, a greater proportion of CD4+CD28null T-cells from dialysis patients expressed granzyme B and perforin compared to healthy individuals [177]. Further studies have found CD4+CD28null T-cells to be associated with an increased risk of an atherosclerotic event shortly after renal transplantation [174], as well as increased early atherosclerotic damage defined as impaired flow-mediated vasodilatation and increased intima-media thickness in the carotid artery [173, 176, 178-181]. Interestingly, in one study the size of the CD4+CD28null T-cell expansion was negatively correlated with serum vitamin D levels in agreement with the findings of Liaskou *et al.* in PSC patients [176, 218].

Finally, in renal transplant recipients CD4+CD28null T-cells were associated with delayed graft function and reduced renal function, were found to express CX3CR1, NKG2D and cytolytic molecules and were capable of inducing glomerular endothelial cell apoptosis *in vitro* in an NKG2D dependent fashion [225].

1.2.5.4 CD4+CD28null T-cells in cardiovascular disease

The relevance of CD4+CD28null T-cells in cardiovascular disease was first noted in 1999 when Liuzzo *et al.* compared cytokine production by T lymphocytes in patients with unstable angina versus stable angina. They found a persistent increase in the proportion of CD4 and CD8 T-cells that produced IFN-γ in response to phorbol myristate acetate (PMA) and ionomycin and that this increased production of IFN-γ was attributed to an expansion of CD4+CD28null T-cells in patients with unstable angina [226]. The same investigators then showed that CD4+CD28null T-cells are preferentially found in unstable rather than stable atherosclerotic plaques suggesting a direct involvement of this cytotoxic subset in plaque disruption. They also found that CD4+CD28null were highly oligoclonal and that clonotypes of different patients shared T-cell receptor sequences implicating chronic stimulation by a common antigen [227]. In addition, CD4+CD28null T-cells were shown to possess the ability to lyse human umbilical vein endothelial cells (HUVECs) in *in vitro* cytotoxicity assays where cytolysis was dependent on granule exocytosis and augmented by pre-treatment of HUVECs with C-reactive protein (CRP) [228]. Since then several researchers have reported on associations between CD4+CD28null T-cell expansions and increased cardiovascular disease in the context of inflammatory disorders, diabetes and established ischaemic heart disease as already discussed [171, 173-175, 179, 180, 184, 205, 229] whilst CX3CR1 expressing CD4+CD28null T-cells linked to atherosclerotic damage have been described in RA and CKD patients [179, 230].

1.2.6 Origin of CD4+CD28null T-cells

There is no current consensus in the literature as to the exact origin of CD4+CD28null T-cells [171, 184]. The oligoclonal nature of CD4+CD28null T-cells points to a persistent antigen as the driver behind their expansion and accumulating evidence now suggests that the bimodal distribution of CD4+CD28null T-cells can be explained by previous infection with CMV. In a cohort of RA patients CD4+CD28null T-cells were only expanded in CMV seropositive patients [168, 231]. These findings were confirmed in MS patients [159] and renal transplant recipients [225]. Similarly in AAV significant expansions of CD4+CD28null T-cells are only found in CMV seropositive patients [112, 224]. Furthermore, Morgan et al. showed that once CMV seropositivity was taken into account there was no difference in CD4+CD28null T-cell percentage between AAV patients and age-matched healthy volunteers [112].

Further support for CMV as the driver behind CD4+CD28null T-cells came from a study of renal transplant recipients where following primary CMV infection CD4+CD28null T-cells capable of producing granzyme B and perforin emerged after cessation of the viral load indicating that CMV triggers their formation [232]. However it remains unclear as to what mechanisms are responsible for the maintenance and / or expansion of CD4+CD28null T-cells following primary CMV infection and during latency.

An intriguing alternative hypothesis for CD4+CD28null T-cell reactivity has also been proposed. Zal *et al.* showed that CD4+CD28null T-cells from patients with acute coronary syndrome (ACS), but not from stable angina or healthy volunteers, recognised heat shock protein 60 (HSP60) as an antigen [200]. This association was confirmed in another study that found increased expression of IFN-γ, granzyme B and perforin when CD4+CD28null T-cells from CKD patients were incubated with HSP60 or HSP70 whilst again there was no reactivity in CD4+CD28null T-cells from healthy volunteers to these antigens [178].

Heat shock proteins can be expressed by all cells under certain conditions of stress such as oxidative stress or inflammation including exposure to proinflammatory mediators such as IFN-γ and TNF-α [184, 233] potentially explaining this association. Furthermore, although there is some evidence for HSP60 being involved in atherogenesis in that high levels of autoantibodies specific for HSP60 have been associated with cardiovascular disease [234], the presence of high levels of HSP70 in serum is associated with protection against cardiovascular disease rather increased risk [233, 235]. Interestingly, an HSP60 epitope has recently been found to display homology with two human CMV-encoded proteins, UL122 and US28 [236]. In that study, patients with coronary artery disease were found to have circulating antibodies specific for this HSP60 epitope as well as the two homologous CMV proteins whereas healthy volunteers did not. In addition, US28 is a chemokine receptor homologue encoded by CMV that has been established as a key mediator of CMV mediated

vascular pathology through its binding to several human chemokines including fractalkine [237]. As such, infection with CMV may lead to the induction of a cascade of immune responses leading to endothelial damage partly through molecular mimicry involving HSP60 [233] potentially explaining the observed reactivity of CD4+CD28null T-cells to heat shock proteins.

1.3 Cytomegalovirus

1.3.1 CMV epidemiology

Human cytomegalovirus (CMV) is a common double stranded DNA beta herpes virus that has co-evolved with its human host over millennia. Approximately 60-90% of the population worldwide is infected with CMV [238-241]. CMV is almost ubiquitous in developing countries in South America, Africa and Asia whereas in Western Europe and the United States the prevalence of infection increases with age with approximately 90% of individuals older than 90 years being infected compared to 40-60% amongst middle aged people and is also significantly influenced by socioeconomical status [242-246]. Viral spread occurs through bodily fluids or vertical transmission and CMV remains a leading cause of congenital infections associated with a significant risk of developmental birth defects [243]. Furthermore, CMV is an important cause of opportunistic infection in immunocompromised individuals such as bone marrow and solid organ recipients and AIDS patients [247].

1.3.2 CMV infection and the host response

Following primary infection CMV establishes lifelong persistence through a latency phase from where the virus can be reactivated [246]. During acute infection the main target cells for CMV are epithelial and endothelial cells. CMV can cause lytic infection in any cell type, in this way leading to organ specific symptoms and disease. Latency however is mainly established in bone marrow and peripheral immature cells of

myeloid lineage [243, 248-252]. Acute CMV infection can be due to primary infection or reactivation, is characterised by constitutional symptoms and is usually subclinical although organ specific signs and symptoms can occur such as hepatitis, colitis, pancytopenia, retinitis or pneumonitis particularly in immunocompromised individuals [253].

The host response to CMV involves both innate and adaptive immune mechanisms [254]. NK cells have an important role in the immediate control of acute CMV infection [255]. Humoural and cellular mechanisms are subsequently triggered in order to control primary CMV infection [256]. Viral replication is mostly controlled in an immunocompetent host although CMV reactivation has been documented in certain circumstances particularly associated with increasing age [257-259].

The cell mediated response to CMV, primarily by CD8, but also CD4 T-cells, is crucial for successful immune surveillance and control of viral replication, as evidenced by a high rate of reactivation leading to severe clinical sequelae in patients with profound cellular immunodeficiency [260]. Patients receiving allogeneic bone marrow stem cell transplant grafts for the treatment of haematological malignancy are at a particularly increased risk of life threatening CMV reactivation. This is especially the case when a CMV seropositive patient receives a graft that has been T-cell depleted or a graft from a CMV seronegative donor resulting in both cases in lack of specific anti-CMV immunity [261]. Adoptive immunotherapy has been performed by transferring donor derived

CMV-specific CD8+ clones [262, 263] or purified CMV-specific CD8+ T-cells by HLA-peptide tetramer staining followed by selection with magnetic beads [261, 264]. Transfer of virus specific CD8+ T-cells in these studies has been shown to lead to reconstitution of viral immunity, reduction in viral reactivation rates and in a recent clinical trial, clearance of infection refractory to anti-viral therapy [261]. Furthermore, the presence of CD4+ CMV-specific T-cells has been shown to be important for the persistence of transferred CD8+ T-cells [263].

1.3.3 CMV leads to systemic dysregulation of the immune system

CMV is increasingly been recognized as the most immunodominant infection encountered by the human immune system with up to 10% of the CD4 and 40% of the CD8 compartments being comprised of CMV specific T-cells [265]. In states of immunosuppression this is further increased [112] and may indeed reflect increased episodes of CMV reactivation.

CMV infection in healthy individuals substantially modulates the peripheral lymphoid cell pool leading to the accumulation of effector memory (EM) (CD45RA- CCR7- CD28-) and late-stage differentiated (CD45RA+ CCR7- CD28-) CD8 and CD4 T-cells at the expense of a reduction in naïve T-cells (CD45RA+ CCR7+ CD28+) [266], a phenomenon that has been termed ‘memory inflation’ [267, 268]. The presence of large numbers of EM CMV-specific T-cells suggests recent encounter with viral antigen. The highly

differentiated EM T-cells that accumulate following CMV infection are potent producers of proinflammatory cytokines such as TNF-α [269]. Interestingly, it has recently been shown that TNF-α can reverse the transcriptional silencing that keeps CMV in latency thereby forcing the virus out of latency into the lytic phase leading to reactivation [270]. CMV driven expansion of proinflammatory T-cells may therefore in this way lead to a positive feedback loop of further CMV reactivation although this is currently unclear.

1.3.4 CMV is associated with inflammation and vascular pathology

CMV has been repeatedly associated with an augmented inflammatory response [271-273]. In a study of ESRD patients, CMV seropositivity was associated with inflammation, lower haemoglobin levels and a demand for a higher erythropoietin dosage that was also correlated to the size of the CD4+CD28null T-cell expansion [274].

Several studies have suggested a link between CMV exposure and vascular pathology including cardiac allograft vasculopathy [275], increased mortality due to CVD in the context of renal transplantation [276, 277], atherosclerosis [278] and CVD in the general population [279]. In addition, CMV has been implicated in the development of hypertension [280, 281].

A study examining atherosclerotic plaques from 105 coronary artery bypass grafting (CABG) surgical patients found that the presence of CMV replication in plaques was linked to a 4-fold increase in the history of previous acute coronary syndrome after adjustment for other risk factors [282]. CMV may also contribute to a higher risk of atherosclerotic events following renal transplantation where pre-transplantation CMV exposure has been shown to be an independent risk factor for cardiovascular episodes in the post-transplantation period. Furthermore the presence of actual CMV replication following transplantation further increases this risk [283].

High CMV IgG antibody titres have been independently associated with systolic as well as diastolic blood pressure elevation and flow mediated vasodilation in young Finnish men [284] suggesting that latent CMV infection may lead to adverse vascular changes from a young age and may be linked to the development of arterial stiffness, an established surrogate marker of CVD risk. Indeed a recent study from Birmingham has shown an association between CMV seropositivity and increased arterial stiffness in patients with chronic kidney disease [285].

Furthermore, it has been shown that the host inflammatory response to chronic CMV infection is linked to immune-pathological pathways leading to vascular and endothelial injury. Specifically the CD4 T-cell response to CMV can drive the expression of fractalkine on endothelial cells that are then susceptible to attack by monocytes and NK cells bearing the fractalkine receptor CX3CR1 [286]. Furthermore

CD4+CD28null CMV specific T-cells have been found to express high levels of CX3CR1 and CXCR3 and *in vitro* and are able to migrate in a fractalkine and IP-10 dependent fashion towards activated endothelial cells inducing endothelial cell apoptosis [287], implicating CMV as the driving force behind the observed association between CD4+CD28null T-cells and cardiovascular disease. Further confirmatory evidence has come from a recent study in octogenarians that found CMV seropositivity as well as expansions of CD4+CD28null T-cells to be associated with overall cardiovascular mortality as well as death from myocardial infarction and stroke [279].

1.3.5 CMV is associated with immunosenescence

Recent epidemiological evidence has linked CMV infection to an increased risk of morbidity and mortality in the elderly [267, 288-294]. CMV is thought to contribute to immunosenescence [288] and constitutes part of the 'immune risk profile' (IRP) comprised of an inverted CD4 to CD8 ratio of < 1, expansion of CD8 T-cells with a late-stage differentiation phenotype, a low frequency of naïve T-cells, poor proliferative T-cell responses, an increase in proinflammatory cytokines and CMV seropositivity [291]. CMV seropositivity is associated with a CD4:CD8 ratio of < 1, poor cognition and functional disability in older adults [295].

Abnormalities of the CD4 T-cell compartment are also increasingly noted with an increase in CD4 CMV-specific cells with age, leading to an expansion of CD4+CD28null

T-cells and a reduction in CD4 naïve T-cells [265, 296]. Furthermore, CMV infection may impair the body's ability to fight heterologous infections such as Epstein Barr virus (EBV) [297], herpes simplex [298] and herpes zoster [299] and leads to decreased antibody responses following influenza vaccination [300].

Several studies have now linked chronic CMV infection to reduced responsiveness to the influenza vaccine. In these studies a higher degree of inflammation (increased concentration of IL-6 and TNF-α) amongst CMV seropositive patients as well as higher levels of anti-CMV IgG correlated with poor vaccine responses. Importantly high frequencies of terminally differentiated CD4+CD28null T-cells were persistently associated with reduced vaccine-induced antibody responses [300-302].

1.3.5.1 CMV and immunological functional exhaustion

In addition to memory inflation, functional exhaustion of CMV-specific cells may contribute to CMV driven immunosenescence by modulation of cytokine production over time leading to loss of function. Following repeated antigen stimulation with viruses, such as HIV, cytokine production is progressively reduced in a hierarchical manner. IL-2 production and cellular proliferative capacity is lost first, followed by TNF-α and finally IFN-γ production [303]. In a study of elderly CMV seropositive patients, up to 50% of CMV-specific CD4 T-cells were unable to produce IL-2 and only capable of IFN-γ production [304]. Furthermore, loss of IL-2 production by CMV-

specific CD4 T-cells is associated with an increased risk of CMV end-organ disease in CMV seropositive individuals infected with HIV [305].

Associated with cytokine modulation is an increase in the expression of inhibitory receptors cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3) and lymphocyte activation gene 3 (LAG-3), as well as up-regulation of the transcription factor PR domain zinc finger protein 1 (BLIMP-1), thereby leading to attenuation of virus specific T-cell function [303]. In HIV seropositive patients [306] and in transplant patients with CMV viraemia [307], inhibitory receptors are expressed on CD4 CMV-specific cells, particularly CD4+CD28null T-cells, although to a lesser extent than found on CD4 HIV-specific T-cells [306]. These findings were confirmed during primary CMV infection in pregnant women where CMV specific T-cells were found to have a reduced capacity for proliferation, decreased production of IL-2 and increased expression of PD-1 [308].

On the other hand, recent studies have suggested that in younger people, CMV may confer immune protection against other pathogens. For instance, CMV has been shown to augment immune responses to heterologous pathogens in young mice whereas it impairs immunity to other viruses in older mice [309-313]. CMV seropositive young adults exhibit enhanced antibody responses to influenza vaccination compared to CMV seronegative individuals [314] and in a study of Gambian infants, CMV infection was shown to enhance some immune responses in

infancy despite inducing an expansion of CD8 and CD4 late differentiated cells [315]. Finally, Pera *et al.* have recently suggested that in young individuals (18-35 years old), CMV induces the expansion of a polyfunctional subset of CD4 T-cells co-expressing CD57 and CD154.

The potential advantage conferred by past CMV infection in dealing with heterologous antigens during early life makes logical sense from an evolutionary perspective. Nevertheless, although the CMV induced expansion of proinflammatory T-cells may be protective to begin with, with advancing age and in the setting of inflammatory pathology, it is possible that inflammation drives a vicious circle of persistent CMV reactivation followed by further expansion of proinflammatory T-cells leading to the observed association between CMV and inflammatory pathology including cardiovascular disease.

In terms of the role of CMV in immunosenescence, recent studies have continued to support the notion that CMV has a negative impact on cellular immunity to heterologous infection such as respiratory viral infections and influenza in older individuals [316, 317]. Furthermore in the study by Pera and colleagues the proportion of CD4+CD28null T-cells in CMV seropositive patients was inversely associated with the percentage of polyfunctional CD4+CD57+ T-cells even in young age [309] highlighting the complexities of the footprint of CMV infection on the immune system and suggesting a possible association between proinflammatory CMV specific T-cells and

immunosenescence even early on in life. Finally, genetic factors are likely to also play a significant role in the observed variability of the impact of CMV on T-cell phenotypes [318].

1.3.6 CMV replication in latency and pharmacological suppression

The observed expansion of CMV specific EM cells including CD4+CD28null T-cells in inflammatory conditions and with advancing age suggests repeated exposure to CMV antigen. There are no studies that have specifically assessed the extent of subclinical CMV reactivation in AAV. However, a cross-sectional study of 18 patients with inflammatory connective tissue diseases such as SLE and RA on similar immunosuppressive therapy used in AAV, detected CMV reactivation in up to 41% of patients [319]. Furthermore, another study has detected CMV DNA in the urine of 90% of elderly individuals over a 6-month period supporting frequent sub-clinical CMV reactivation with advancing age [257].

Valaciclovir, the prodrug of aciclovir, inhibits viral DNA polymerase and is incorporated in viral DNA leading to chain termination. It has activity against several human viruses including CMV. Clinical trials in AIDS, bone marrow transplantation and solid organ transplantation have demonstrated that valaciclovir treatment leads to reduced viral load in blood and urine in AIDS patients [320, 321] and reduced episodes of clinical CMV disease in transplantation [322]. Prophylactic valaciclovir treatment for 3 months

in kidney transplant patients has been demonstrated to suppress viral replication leading to much lower rates of detectable CMV DNA in the blood compared to control patients [323]. In addition, treatment of CMV viraemia reduces PD-1 expression and increases IL-2 production in renal transplant recipients [307].

To date there have not been any human interventional studies to determine whether treatment with an anti-viral agent can reduce subclinical CMV reactivation and whether this can ameliorate the negative effects of CMV on the immune system. Beswick *et al.* however have recently shown that valaciclovir therapy can potentially reverse the development of immunosenescence in elderly mice seropositive for murine CMV [324]. In this study, valaciclovir treatment for up to 12 months reduced the magnitude and differentiation of CMV-specific CD8 T-cells, increased the proportion of naïve CD8 T-cells and led to a reduction in influenza A virus loads following an influenza challenge.

1.4 Summary and hypothesis

Expansions of proinflammatory CD4+CD28null T-cells have been repeatedly reported in inflammatory conditions including AAV as well as in patients with ischaemic heart disease and older individuals. Significant expansions of CD4+CD28null T-cells are only seen in CMV seropositive individuals, and in AAV, CD4+CD28null T-cells are associated with increased infection and higher mortality.

In addition, CMV seropositivity and its association with an expansion of EM T-cells, including CD4+CD28null T-cells, is linked to immunosenescence and vascular pathology. This is highly relevant in AAV where the two leading causes of morbidity and mortality are infection and cardiovascular disease. However, the phenotype of CD4+CD28null T-cells in AAV has not been adequately described and it is currently unknown as to whether expansions of CD4+CD28null T-cells contribute to CVD risk in AAV.

The mechanisms that drive the expansion of CD4+CD28null T-cells in CMV seropositive individuals are currently unclear. It is also not known as to whether such CMV induced modulation of the immune system in inflammatory diseases can be reversed. Furthermore, there is some controversy in the literature in that alternative antigens have been postulated to be responsible for the expansion of CD4+CD28null T-cells.

In addition, the cytokine production and inhibitory receptor profile of CMV specific CD4+CD28null T-cells in patients with expansions of this subset have not yet been addressed. Finally, the relationship between these features and control of CMV replication *in vivo* is also unknown.

The hypothesis of the research presented here is that subclinical CMV reactivation in AAV drives the expansion of proinflammatory CD4+CD28null T-cells giving rise to adverse modulation of the immune system that progressively leads to vascular damage and immunosenescence thereby explaining the observed increase in infection and mortality seen in association with increased expansions of CD4+CD28null T-cells in AAV and that blocking subclinical CMV reactivation with valaciclovir will reduce expansion of this subset.

1.4.1 Thesis aims and objectives

The aims of this thesis were to characterise the ways in which CMV negatively impacts on the immune system in inflammatory conditions and determine whether this adverse modulation can be reversed using AAV as a model of inflammatory disease.

The specific objectives of the research conducted were to:

- Determine the phenotype of CD4+CD28null T-cells in AAV and compare this to age matched healthy volunteers
- Investigate whether CMV specific CD4+CD28null T-cells in AAV are functionally exhausted by characterising their cytokine and inhibitory receptor expression and explore whether this has a bearing on the control of subclinical CMV reactivation
- Determine the impact of CD4+CD28null T-cells on CVD risk in AAV by assessing their relationship to arterial stiffness, an established marker of CVD risk
- Determine whether subclinical CMV reactivation drives the expansion of CD4+CD28null T-cells by conducting a proof of concept randomised controlled clinical trial in AAV patients, and finally
- Explore whether pharmacological therapy can halt or reverse the adverse modulation of CMV on the immune system by blocking subclinical CMV reactivation in AAV patients.

CHAPTER 2

METHODS

Chapter 2 Methods

2.1 Study Design Overview

The aims and objectives of this research study were discussed in Chapter 1. Central to the work undertaken during this PhD was the design and implementation of a proof of concept randomised controlled clinical trial. The aim of the clinical trial was to test whether treatment with an oral anti-viral agent, valaciclovir, over 6 months can control subclinical CMV reactivation in AAV and in turn whether this can ameliorate CMV induced changes on the immune system. The main objective of the clinical trial was to test the hypothesis that subclinical CMV reactivation leads to expansion of CD4+CD28null T-cells and that by blocking subclinical CMV reactivation the expansion of this cell subset can be halted or reduced. The clinical trial arm of the study aimed to randomise 50 CMV seropositive AAV patients in disease remission, in an open label design to valaciclovir treatment for 6 months, versus no additional therapy. In order to fully address the rest of the research study objectives, a cross-sectional study arm was also set up where additional AAV patients meeting the clinical trial eligibility criteria but that did not want to participate in the clinical trial, could be recruited for a one-off visit and undergo the same assessments and investigations that were performed on the clinical trial patients at their baseline visit. Furthermore, the cross-sectional arm of the study aimed to recruit a total of 30 CMV seropositive healthy volunteers for an identical one-off visit in order to compare CMV induced changes on the immune system between AAV patients and healthy volunteers (Figure 2.1).

2.2 Clinical Trial Arm Design and Implementation

The study protocol (Version 1.1) was approved by the Research Ethics Committee (REC) of Yorkshire & The Humber – Leeds West (REC reference 12/YH/0377) on the 21st of August 2012. Approval was also gained by the University Hospital Birmingham (UHB) Research Governance Office (RnD) (Project reference RRK 4456) and the Medicines and Healthcare Products Regulatory Agency (MHRA) on the 20th of November 2012 (Eudract Number: 2012-001970-28).

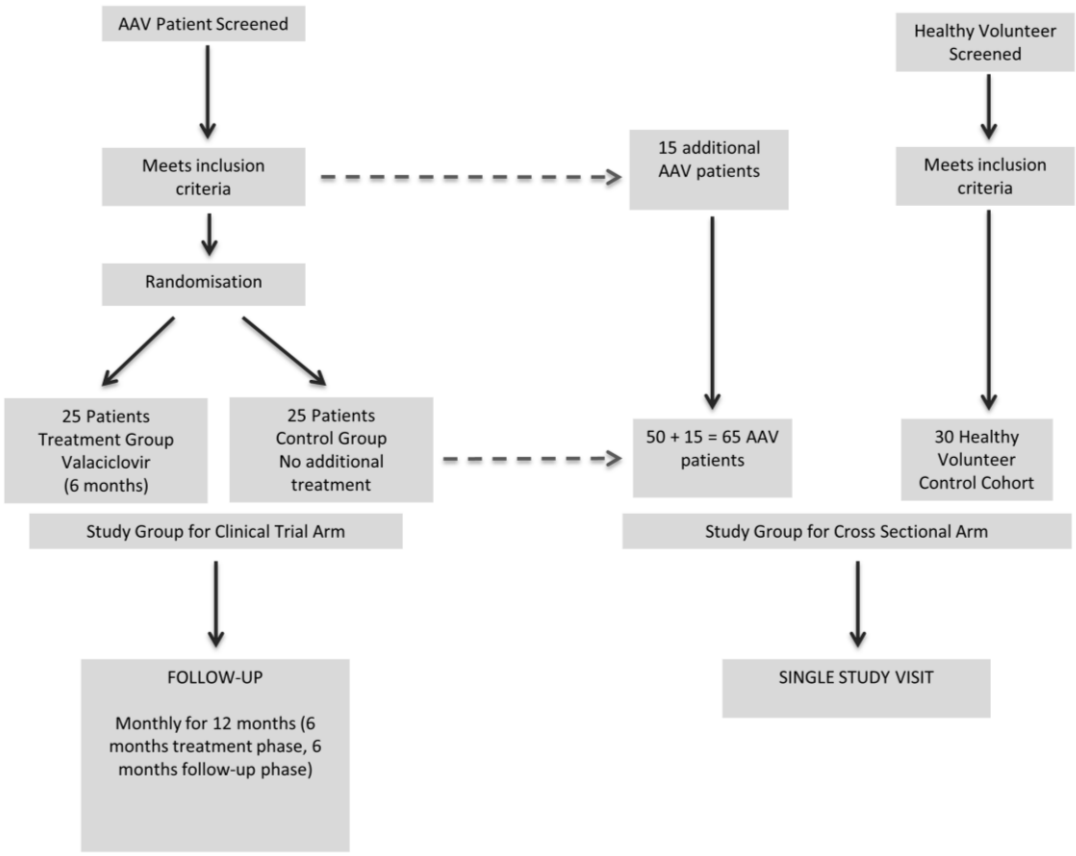

Figure 2.1 Study design diagram

Clinical Trial Arm is shown on the left and the Cross-Sectional Arm of the study on the right side.

A total of six substantial amendments to the study protocol were submitted and approved by the REC, UHB Governance Office and the MHRA where appropriate. The clinical trial was registered with ClinicalTrials.gov (Identifier NCT01633476). The following section describes the clinical trial protocol and is based on the final version of the study protocol (Version 3.0; Appendix 2). Section 2.3 is an excerpt of the trial protocol paper as published by *Trials* Journal [325] with minor formatting and explanatory modifications. The author of this thesis was the first author in this paper and was extensively involved in the study design, applications for REC, MHRA and NHS Research and Development approvals and amendment approvals, study coordination, patient recruitment and drafting and final submission of the manuscript.

2.3 Valaciclovir to prevent Cytomegalovirus (CMV) mediated adverse modulation of the immune system in ANCA associated VASculitis (CANVAS): Study protocol for a randomised controlled clinical trial

2.3.1 Design

This was a single-centre, open label, proof of concept, randomised controlled trial of oral valaciclovir treatment (2g four times a day; reduced appropriately depending on renal function) for 6 months versus no additional treatment, in CMV seropositive AAV patients in remission, followed by a 6 month follow up period. No placebo was used.

The primary outcome was the proportion of patients with CMV reactivation, as assessed by measurable viral load on quantitative blood and urine CMV polymerase chain reaction (PCR). The secondary outcomes were safety, as defined by adverse events sufficient to stop treatment with valaciclovir, change in the proportion of CD4+ CMV specific T-cell population (defined for the purposes of the clinical trial as CD4+CD28null cells) and change in soluble markers of inflammation from baseline to 6 months (Table 2.1). Further tertiary and exploratory outcomes are listed in Table 2.1 and included persistence of the effect of valaciclovir on the proportion of CD4+CD28null cells at 6 months post completion of treatment, change in the immune phenotype of CD4+ T-cells, change in CMV IgG titre and change in blood pressure and arterial stiffness parameters from baseline to 6 months.

Table 2.1 Clinical Trial Endpoints

	Description	Specific measurement variable
Primary outcome		
	Proportion of patients with CMV reactivation	Quantification of viral DNA copies in blood and urine by quantitative polymerase chain reaction (qPCR)
Secondary outcomes		
	Safety	Number of adverse events and incidence of events by system organ class
		Adverse events sufficient to stop treatment with study drug
	Change in the proportion of CD4+CD28null cells from baseline to 6 months	Proportion of CD3+CD4+CD28null T cells in peripheral blood
	Change in the concentration of soluble markers of inflammation from baseline to 6 months	Concentration of IL-2, tumour necrosis factor alpha (TNF-α), IFN-γ, IL-6, IL-10, IL-17 and highly sensitive C-reactive protein (CRP) in peripheral blood
Tertiary outcome		
	Persistence of valaciclovir effect on the proportion of CD4+CD28null cells from 6 months to 12 months	Proportion of CD3+CD4+CD28- T cells in peripheral blood
Exploratory outcomes		
	Change in the absolute count of CD4+CD28null T-cells from baseline to 6 months	Absolute count of CD3+CD4+CD28null T-cells
	Change in other associated T-cell subsets from baseline to 6 months	Proportion of CD4+CD28null cells secreting IFN-γ in response to CMV lysate stimulation
		Proportion of CD3+CD4(-)CD28null T cells in peripheral blood
	Change in CMV IgG antibody titre	CMV IgG titre
	Change in soluble markers of endothelial damage from baseline to 6 months	Concentration of fractalkine, IP-10, regulated on activation, normal T cell expressed and secreted (RANTES), P-selectin, E-selectin, monocyte chemoattractant protein-1 (MCP-1), soluble vascular cell adhesion molecule 1 (sVCAM-1) and soluble intracellular cell adhesion molecule 1 (sICAM-1) in peripheral blood
	Change in arterial stiffness baseline to 6 months	Carotid to femoral pulse wave velocity

The trial sponsor was the University of Birmingham and the study was delivered through the National Institute for Health Research (NIHR) / Wellcome Trust (WT) Clinical Research Facility (CRF) at the University Hospital Birmingham (UHB) NHS Foundation Trust that also hosts the UHB Vasculitis Clinic. The trial was funded by the Wellcome Trust and Vasculitis UK. The trial was coordinated by the Trial Management Group (Table 2.2) according to the current guidelines for Good Clinical Practice (GCP) and ensuring protection of patients' rights as detailed in the Declaration of Helsinki. All laboratory assays were carried out in laboratories that fulfil the principles of Good Laboratory Practice and assays informing the primary and secondary outcomes were fully validated prior to study commencement (see Section 2.7). The protocol was designed based on the SPIRIT guidelines [326-328].

Table 2.2 Trial Management Group (TMG)

	Role	Institution
Professor Lorraine Harper	Principal Investigator	Institute of Clinical Sciences, University of Birmingham
Dr Dimitrios Chanouzas	Co-Investigator Analytical Project Manager	Institute of Inflammation and Ageing, University of Birmingham
Dr Matthew Morgan	Co-Investigator	Institute of Clinical Sciences, University of Birmingham
Professor Paul Moss	Co-Investigator	Institute of Immunology and Immunotherapy, University of Birmingham
Dr Peter Nightingale	Statistical Advisor	Wolfson Computer Laboratories, University Hospital Birmingham
Sister Linda Coughlan	Research Nurse	NIHR Clinical Research Facility, University Hospital Birmingham
Sister Annabel Grinbergs	Research Nurse	NIHR Clinical Research Facility, University Hospital Birmingham

2.3.2 Participants

CMV seropositive patients with AAV in stable remission for 6 months or longer and on a maximum of two immunosuppressant agents were recruited from the Vasculitis Clinic at UHB NHS Foundation Trust. Recruitment took place over 15 months. Patients were approached at routine clinic visits. Patients could also be contacted via post by sending them a copy of the Patient Information Sheet (PIS; Appendix 3). Patients were

allowed a minimum of 24 hours to reflect on the content of the PIS before informed written consent was obtained by completion and signing of the study specific informed consent form (ICF; Appendix 3).

A full list of study inclusion and exclusion criteria is given in Table 2.3. In addition, patients on TNF inhibitors or subcutaneous immunoglobulin (Ig) therapy were not eligible for inclusion into the trial as TNF inhibitors can lead to re-expression of CD28 on CD4+CD28null T-cells [215] and subcutaneous Ig treatment can interfere with the production and relative concentration of anti-CMV IgG antibodies [329]. Subjects were withdrawn from the trial if they chose not to continue or the investigators felt that continued participation in the trial was inappropriate. Subjects who withdrew from the intervention were asked if they would be prepared to continue to attend follow up clinics. The study's primary and main secondary outcomes were based on objective laboratory assays, therefore minimising the risk of performance bias in this open-label design. Furthermore, treatment and medical management other than valaciclovir was identical between the treated and control patients.

Table 2.3 Clinical Trial Inclusion and Exclusion Criteria

Inclusion criteria	
	Documented diagnosis of granulomatosis with polyangiitis (Wegener's), microscopic polyangiitis or renal limited vasculitis according to Chapel Hill Consensus Conference Criteria
	In stable remission (no documented clinical disease activity) for at least 6 months prior to study entry
	On maintenance immunosuppression with prednisolone, mycophenolate mofetil or azathioprine alone or in combination (maximum two agents)
	Documented past evidence (any time point) of CMV infection (CMV-specific immunoglobulin G detected in peripheral blood)
	Documentation that female patients of child-bearing potential are not pregnant and are using an appropriate form of contraception
	Written informed consent for study participation
Exclusion criteria	
	Stage 5 CKD (estimated glomerular filtration rate (eGFR) <15 mL minute^{-1} 1.73 m^{-2}); tests performed within 6 months of pre-baseline visit can be used for this assessment
	Other significant chronic infection (HIV, hepatitis B, hepatitis C or tuberculosis)
	B-cell depleting therapy within 12 months or T-cell depleting therapy within 6 months
	Treatment with TNF inhibitors or treatment with intravenous or subcutaneous immunoglobulin within 6 months
	Treatment with anti-CMV therapies in the last month
	Underlying medical conditions, which in the opinion of the investigator place the patient at unacceptably high risk for participating in the study
	Inability to participate fully or appropriately in the study

2.3.3 Schedule of Assessments

Patients attended for a total of 14 visits over a period of just over 12 months (Figure 2.2). Evaluation of CMV reactivation by deoxyribonucleic acid (DNA) PCR of blood and

urine was done monthly. Evaluation of the tolerability of the drug and adverse events occurred monthly. Immune assessments as detailed below and in Table 2.1 were performed at entry, 6 months and 12 months (Figure 2.2).

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
TIMEPOINT	*Pre-Baseline visit*	Pre-Baseline visit	*M0*	*M1-M5*	*M6*	*M7*	*M8-M11*	*M12*
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
CD4+CD28- assessment	X							
Allocation		X						
INTERVENTIONS:								
Valaciclovir treatment			←	—	→			
ASSESSMENTS:								
Determination of viral DNA via qPCR in blood and urine			X	X	X	X	X	X
Immune assessments			X		X			X
*Safety assessment and safety blood tests**			X	X	X	X		
Arterial stiffness measurements			X		X			X

Figure 2.2 SPIRIT figure showing study's schedule of enrolment, intervention and assessment.

M0: baseline visit; M1 to M12: month 1 to month 12; qPCR: quantitative polymerase chain reaction. * Safety blood tests were only performed on treatment group.

Following informed written consent patients underwent an initial pre-baseline visit. During this visit a 5mL blood sample was drawn that was used to determine the

percentage of CD4+CD28null cells. This value was utilised in the stratification of patients as explained below.

At the baseline, 6 month and 12 month visits, a total of 50mL of blood sample was drawn in addition to a 50mL sample of urine. The samples were used to determine CMV viral copies by DNA PCR in blood and urine (primary outcome), the proportion of CD4+CD28null cells in peripheral blood by flow cytometry (secondary outcome), the concentration of soluble markers of inflammation (Table 2.1) in peripheral blood (secondary outcome) and immune assessments and T-cell phenotyping as detailed in the exploratory outcomes (Table 2.1). Excess sample was stored appropriately. Blood pressure and arterial stiffness measurements were also carried out at the baseline, 6 month and 12 month visits as detailed below.

During the remainder of the monthly visits, a 10mL blood sample was drawn in addition to a 50mL sample of urine used to determine CMV viral copies by DNA PCR in blood and urine (primary outcome). Excess sample was again stored appropriately.

In addition, safety blood tests as defined below were performed monthly for the duration of treatment for those patients randomised to receive the drug.

Appointments were scheduled on a monthly basis with an allowance of 10 days either side of the estimated due date of the monthly appointment in order to allow for patient convenience and flexibility. The overall treatment period (or control period) of 6 months was also subject to the same 10 day rule in order to avoid excessive movement of the length of treatment.

2.3.4 Study Assessments

Enumeration of CMV DNA copies for the determination of the primary outcome (CMV reactivation) was carried out by PCR of plasma and urine. This assessment was performed by the UHB Virology Laboratory using an existing validated assay utilised for clinical samples.

Whole blood was stained with fluorochrome conjugated monoclonal antibodies to CD3, CD4 and CD28 and analysed via flow cytometry (LSR II Flow Cytometer, DIVA Software; BD) in order to determine the proportion of CD4+CD28null cells in peripheral blood. Plasma was assessed via luminex technology for the determination of soluble markers of inflammation (Table 2.1). These assays were validated for precision and reproducibility prior to commencement of the trial (Section 2.7).

Blood pressure and arterial stiffness determination were conducted using the Vicorder system (Skidmore, Bristol, UK) [330, 331]. Carotid to femoral pulse wave velocity

(cfPWV) was used to estimate arterial stiffness (see Appendix 4 for detailed Work Instruction used). The Vicorder system provides a non-invasive non-operator dependent method of obtaining cfPWV using a volume displacement method. Patients were rested in a supine 30 degrees head tilt position for 5 minutes prior to inflating a 100 mm wide blood pressure cuff on the non-dominant arm to determine peripheral blood pressure. A 30 mm wide partial cuff was placed on the neck at the level of the carotid artery and a 100 mm wide blood pressure cuff placed around the proximal thigh. The distance between the mid-clavicular point and the mid-point of the thigh cuff was measured and entered in the Vicorder instrument as the aortic path length. The neck and thigh cuffs were inflated to 60mm Hg and the Vicorder instrument utilised the resultant oscillometric signal to extract the pulse waveforms and pulse transit time to calculate cfPWV. The mean value of three consistent recordings was used for subsequent analysis. Inconsistent values were re-analysed by a senior independent examiner not involved in taking the measurements to determine the validity of each measurement.

2.3.5 Randomisation

Randomisation was performed using the University of Birmingham Primary Care Clinical Research and Trials Unit's (PC-CRTU, fully accredited by the NIHR as a trials unit) independent telephone based randomisation system. Block randomisation by CD4+CD28null cell percentage stratification was used (cut off 40%). The randomisation

used mixed blocks of random size (two, four or six), not known to the research team, therefore, minimising any risk of selection bias.

2.3.6 Treatment

Patients randomised to the treatment arm received valaciclovir orally at 2g four times a day. The dose was reduced appropriately depending on renal function (Table 2.4). The study was open label and valaciclovir was used 'off the shelf' with no modifications to the packaging or labelling of the product. Patients were asked to return unused tablets at each monthly visit in order to monitor compliance with medication. Patients randomised to the control arm received no additional treatment. Safety blood tests were conducted monthly for the duration of treatment for the patients randomised to valaciclovir and comprised of full blood count (FBC), urea and electrolytes (U+E) and liver function tests (LFT). In the event of toxicity (scored using the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0) of grade 2 or less, the adverse event was discussed with one of the Investigators to determine whether drug administration should be temporarily withdrawn or the dose reduced. In the event of toxicity of grade 3 or more, the adverse event was discussed with the Principal Investigator (PI). In such a case the expectation was to withdraw drug administration for one week unless the adverse event was judged by the PI to be unrelated to the study drug. Re-introduction of the drug was based on clinical review. The collection and reporting of data on adverse events and serious adverse events was in accordance with EU Directive 2001/20/EC and UK Legislation.

Table 2.4 Dose modification of valaciclovir according to creatinine clearance

Creatinine clearance (CrCl mL/min)[a]	Valaciclovir dose
>75	2 g four times a day
51–75	1.5 g four times a day
26–50	1.5 g three times a day
10–25	1.5 g two times a day

[a]Tests performed within 6 months of the pre-baseline visit could be used for this assessment

2.3.7 Sample Size Calculation and Planned Statistical Analyses

In an immunocompetent elderly population CMV reactivation occurred in 90% at 6 months [257]. It was expected that CMV reactivation would be at least that in the AAV population as they are significantly immunosuppressed. Information for dosing regimens using antiviral prophylaxis in renal transplant recipients has demonstrated over 90% suppression of CMV reactivation [323]. The trial sample size assumed 90% reactivation in the control limb and a conservative estimate of 50% reactivation in the treated group. The estimated sample size was 50 patients, 25 patients in each arm, based on 80% power at a significance level of $p<0.05$ (two tailed test). The UHB tertiary referral vasculitis clinic has more than 200 patients under long-term follow up. Approximately 90% of patients are in remission at any one time and 70% are seropositive for CMV. CMV status is checked routinely on the first patient attendance in clinic. It was anticipated that 125 patients attending the clinic would be eligible for

the study. Recruitment of 40% would achieve the target of 50 patients. This was a conservative rate given previous experience with patient recruitment at the UHB vasculitis clinic.

All analyses were performed using the intention to treat principle. Baseline covariates were compared between the two arms to observe balance and the success of randomisation. The primary analysis tested the hypothesis that there was no difference in the proportion of patients with CMV reactivation between those receiving antiviral prophylaxis compared with those receiving usual treatment. For secondary outcomes comparisons were made between groups using absolute measures of immune function and change between data at entry and end of treatment within groups. Missing data were dealt with by simple imputation if missing randomly.

A safety analysis was performed on all treated patients. The number of events and incidence of adverse events by system organ class were summarised and relationship to treatment noted.

2.3.8 Trial Management and Monitoring

The trial was coordinated by the Trial Management Group (Table 2.2) and facilitated by the infrastructure provided by the NIHR / WT CRF. A Trial Steering Committee (TSC) that included the TMG as well as two Independent Consultant Nephrologists not

involved in the study or regular review of patients recruited in the trial provided the overall supervision of the trial. The TSC oversaw trial progress, protocol compliance, patient safety and review of updated information. Part of the role of the TSC was to review safety data after the first 10 patients completed treatment in order to ensure that the rate and severity of adverse events was not disproportionate to what would be expected with valaciclovir treatment. As this was a small proof of concept study with a short follow-up, no data monitoring committee was formed, as agreed with the sponsor. Any protocol amendments were submitted to the sponsor and relevant regulatory bodies for approval prior to implementation and trial participants were informed of any protocol modifications.

The University of Birmingham conducted regular monitoring visits in its capacity as the trial sponsor to ensure compliance with the protocol and adherence to GCP and regulations.

The integrity of data entry was ensured using a trial-specific Data Input Quality Control standard operating procedure (Appendix 5). Samples were anonymised and all analyses were undertaken on anonymised datasets with study identifiers replacing personal data. All personal details were kept on NHS secure password-protected servers within UHB NHS Trust. Anonymised data was transferred to password-protected servers in the University of Birmingham for analysis.

2.3.9 Funding Declaration

The majority of this study was funded by the Wellcome Trust following a competitive peer review process as part of a Research Training Fellowship Grant (097962/Z/11/Z). The cost of the study drug was funded by Vasculitis UK. The study was carried out at the National Institute for Health Research (NIHR) / Wellcome Trust Birmingham Clinical Research Facility. The views expressed are those of the author and not necessarily those of the NHS, the NIHR or the Department of Health.

2.3.10 Sponsor

The CANVAS study was sponsored by the University of Birmingham (Dr Sean Jennings, Research Governance and Ethics Manager, Research Support Group, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK). The University of Birmingham holds public liability (negligent harm) and clinical trial (negligent harm) insurance policies, which applied to this trial.

2.4 Clinical Trial Sample Flow and Analysis

The collection and processing of clinical trial samples is shown in detail in Figure 2.3 (see also Laboratory Manual in Appendix 6). Briefly, monthly samples were used to measure the CMV viral copy load in plasma and urine (primary outcome; section 2.6.1) via quantitative polymerase chain reaction (qPCR), as well as estimate the CMV IgG titre in plasma (exploratory outcome; section 2.6.8). Whole blood from the baseline, 6 month and 12 month visits was used to determine the proportion of CD3+CD4+CD28- T-cells (secondary outcome; section 2.6.2), enumerate CD45+CD3+CD4+ T-cells and characterize expression of chemokine receptors and memory markers in a subset of patients via polychromatic flow cytometry. Plasma sample from the baseline and 6 month visits was used to measure soluble cytokines and inflammatory markers using Luminex technology (secondary outcome; section 2.6.3). Peripheral blood mononuclear cells (PBMC) were isolated monthly and cryopreserved in liquid nitrogen (section 2.6.4). This enabled the tracking of the CD4+CD28null T-cell percentage over the 12 month study period in a subset of clinical trial control patients that reactivated CMV during the study (section 2.6.7). Furthermore, at the baseline, 6 month and 12 month visits, following isolation, fresh PBMC were stimulated overnight with CMV lysate or staphylococcal enterotoxin B (SEB) in order to interrogate the phenotype of CMV specific T-cells via flow cytometry with respect to cytokine production and inhibitory receptor expression (exploratory outcome; section 2.6.5).

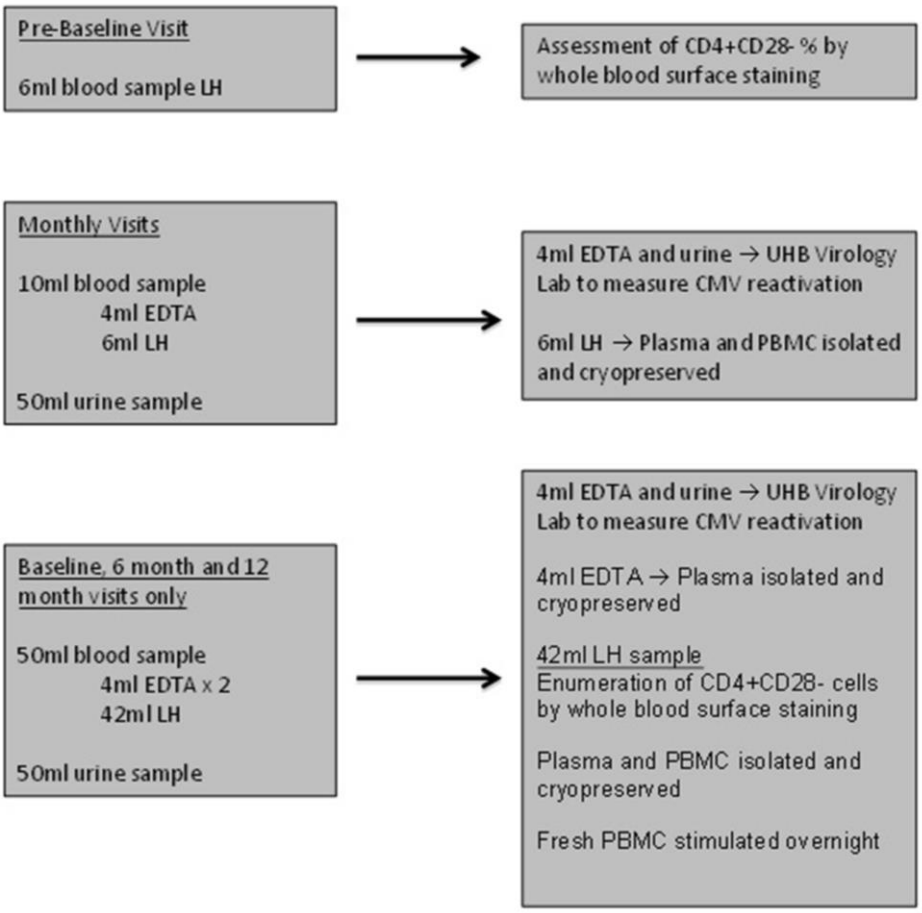

Figure 2.3 Clinical Trial Sample Flow and Planned Analyses
EDTA: Ethylenediamine tetra-acetic acid; LH: Lithium Heparin; UHB: University Hospital Birmingham

2.5 Cross-Sectional Arm Design

As alluded to earlier, the cross-sectional arm of the study was designed to be comprised of a maximum of 65 CMV seropositive AAV patients in remission and 30 CMV seropositive healthy volunteers. The AAV patient group within the cross-

sectional arm was comprised of the clinical trial participants as well as an additional cohort of 15 patients that attended for a single visit. Importantly, the additional AAV patients recruited into the cross-sectional arm fulfilled the same eligibility criteria as the clinical trial patients (Table 2.3). In recruiting the healthy volunteers all efforts were made to match the overall age and gender of the AAV patient and healthy volunteer groups.

Healthy volunteers were identified from non-interventional studies at the NIHR / WT Clinical Research Facility (CRF), friends and family of AAV patients or the research team and the 1000 Elders Cohort (courtesy of Professor J. Lord, Institute of Inflammation and Ageing, University of Birmingham). Past or current systemic inflammatory or auto-immune disease as well as treatment with immunosuppression therapy was an exclusion criterion for HV. Anti-hypertensive medication and asthma steroid inhalers were permissible.

2.5.1 Schedule of assessments

Healthy volunteers were screened in an initial visit where blood was drawn in a lithium heparin tube and plasma assayed for the presence of IgG antibodies to CMV (section 2.6.7). Subsequently, CMV seropositive healthy volunteers and CMV seropositive AAV patients that were recruited directly into the cross-sectional arm of the study attended for a single visit. During this visit the assessments performed were the same as those

performed on the clinical trial patients during their baseline visit (sections 2.3.3, 2.3.4 and 2.4) with the exception that CMV viral load was not measured as preliminary clinical trial data had revealed a relatively low level of CMV reactivation. Blood pressure and arterial stiffness parameters were also assessed as described in section 2.3.4. The data points from the baseline visit of the clinical trial patients were employed for all analyses undertaken within the cross-sectional arm of the study and together with the data points from the additional AAV patients recruited directly into the cross-sectional arm constituted the AAV patient cohort within this arm of the study.

2.6 Laboratory Assays

This section describes the experimental details of the assays employed in the study. All assays that were used to inform the primary and secondary outcomes of the clinical trial arm of the study were formally validated as detailed in section 2.7. All cell culture experiments and flow cytometry staining were performed in 5mL FACS tubes. Centrifuges used were GS-6R (Beckman) and Prism Microcentrifuge (Labnet).

2.6.1 Viral load quantitation

Measurement of CMV viral copies in plasma and urine constituted the primary outcome of the clinical trial arm of the study. For this assay blood was drawn in an EDTA tube and urine was collected in a universal container and sent to the UHB

Virology Laboratory for processing in exactly the same way as clinical samples. There the blood was centrifuged immediately at room temperature for 5 minutes at 3000 rpm (Thermo Megafuge) to isolate the plasma fraction. No additional processing was performed on the urine samples. Samples were then stored at 4^{o}C upright for a maximum of 72 hours before proceeding to determining the number of viral copies per millilitre using a well validated for clinical use qPCR assay (RealTime CMV, Abbot) that the UHB Virology Laboratory employs for all clinical samples.

The Abbott RealTime CMV assay targets 2 short sequences within the UL34 and UL80.5 genes of the CMV genome. These regions are specific for CMV and are highly conserved based on analysis of published CMV sequences [332-334]. The assay is standardised against the 1^{st} World Health Organisation (WHO) International Standard for Human Cytomegalovirus for Nucleic Acid Amplification Techniques (NIBSC 09/162) [335]. Results are reported in this thesis in viral copies / mL. The lower limit for reporting positive results at the UHB virology laboratory is 200 viral copies / mL and therefore this was the lower limit of quantitation used for all primary analyses in this thesis. However the RealTime CMV assay can quantitate down to a lower limit of 20 viral copies / mL and therefore this threshold was utilised in secondary analyses.

The Abbott RealTime CMV assay at the UHB virology laboratory is fully automated using the Abbott m2000sp and Abbott m2000rt instruments. The assay combines PCR technology with homogeneous real time fluorescent detection for the quantitation of

CMV DNA. An unrelated DNA sequence is also introduced into each specimen during sample preparation. This sequence is amplified at the same time as any potential existing CMV sequence and functions as an internal control in order to verify that the assay has been completed successfully for each sample. A positive and a negative control are also included with each assay run.

Briefly, the virions are lysed in a step that includes proteinase K to digest proteins associated with the sample. The nucleic acids are then extracted, concentrated and purified from the sample by the m2000sp automated instrument with the use of magnetic microparticles. The bound nucleic acids are automatically eluted and transferred to a 96 well plate ready for amplification at which point the internal control is also introduced into the sample. The CMV amplification reagent components (CMV Amplification Reagent that contains specific sets of amplification primers for CMV and the internal control, DNA polymerase, and Activation Reagent) are dispensed into the 96 well optical reaction plate along with aliquots of the nucleic acid samples already prepared and following manual application of the optical seal the plate is transferred to the Abbott m2000rt instrument.

There the target DNA is amplified in the presence of deoxynucleotide triphosphates (dNTPs) and magnesium. During each round of PCR amplification, if a suitable target is present the fluorescent probes anneal to the amplified target DNA. Any CMV and internal control amplified sequences are distinguished from each other as their

respective probes are labelled with different fluorescent molecules. The amplification cycle at which fluorescent signal is detected by the Abbott m2000rt is inversely proportional to the log of the CMV DNA concentration present in the original sample. Figure 2.4 shows an example of a positive and a negative for CMV viral copies assay run.

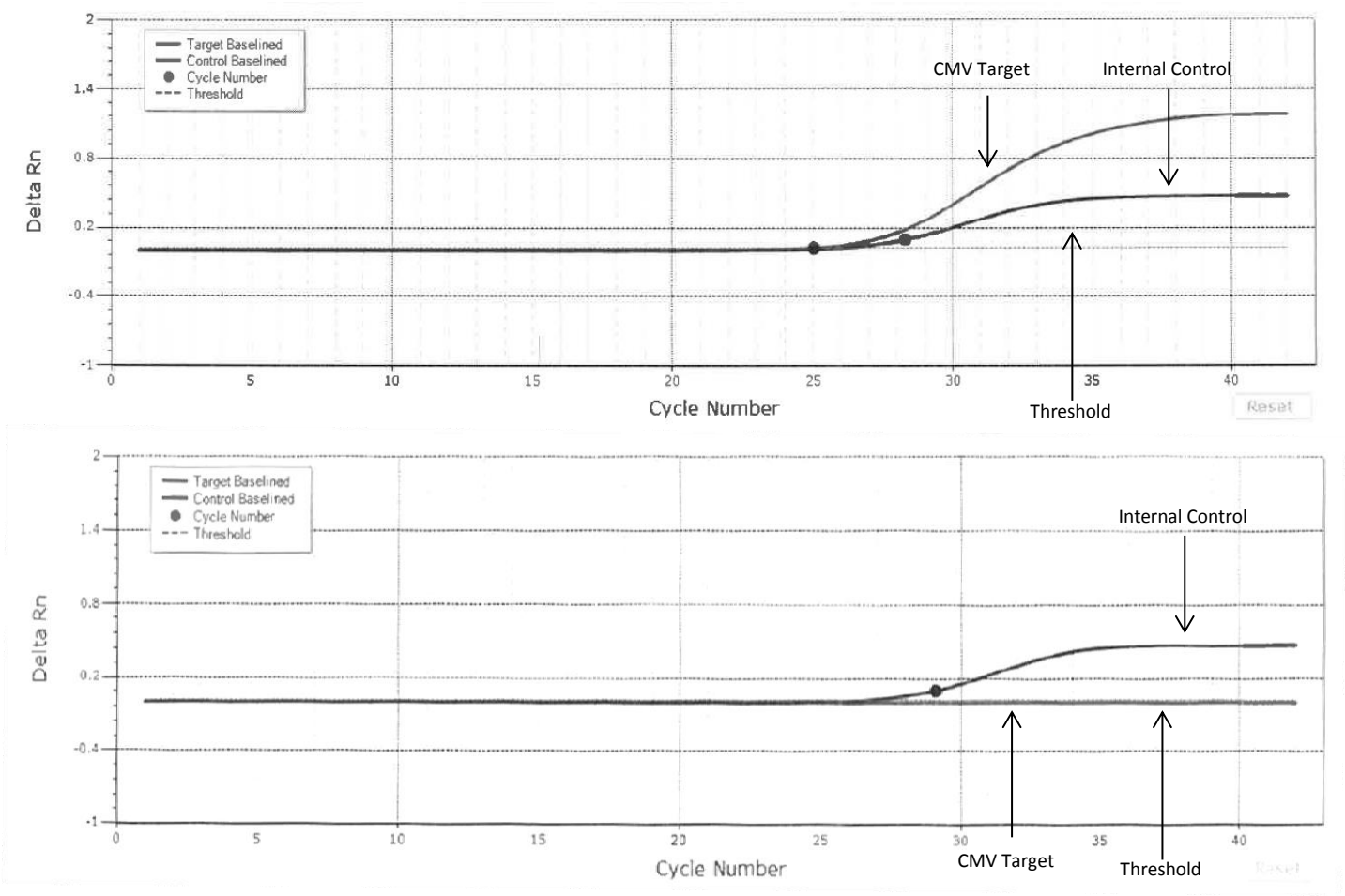

Figure 2.4 Quantitation of CMV DNA in plasma and urine

Quantitation of CMV viral copies in plasma and urine samples for determination of the primary outcome was carried out by the UHB Virology Laboratory using the Abbott RealTime CMV assay. The top panel shows the result of a positive run where CMV target has been detected at PCR amplification cycle 25.03 equating to a CMV viral copy number of 3,840 copies / mL whereas the bottom panel shows the results from an assay run where CMV target was not detected. In both runs the internal control was successfully detected.

2.6.2 Whole blood surface staining of lymphocytes

Whole blood surface staining was used to estimate proportions of CD3+CD4+CD28- T-cells, enumerate CD4 T-cells directly and assess the T helper and memory phenotype of CD4+CD28- T-cells in a proportion of patients. Blood was drawn in lithium heparin (LH) tubes, left upright on the bench at room temperature, and used within a maximum of 5 hours of the draw. Briefly, 100 μL of whole blood, or 50 μL of Cytofix CD4 positive control (see Section 2.7.2), was aliquoted and stained with a freshly prepared monoclonal antibody mix conjugated to appropriate fluorochromes (Table 2.5) for 30 minutes at 4^{o}C prior to incubating for 15 minutes at room temperature with 2 mL of ready to use Red Blood Cell Lysis Buffer (eBioscience). The sample was then centrifuged at 584g for 5 minutes to pellet the cells, the supernatant was decanted and 2 mL MACS buffer (phosphate buffered saline (PBS), 2mM EDTA, 0.5% bovine serum albumin (BSA)) was added to wash the cells. After centrifugation at 584g the cells were re-suspended in 200 μL MACS buffer.

A fluorescence minus one (FMO) control was assayed with each experimental run as an aid for CD28 gating, where the FMO was stained with CD3, CD4 and an isotype control conjugated to the same fluorochrome as the CD28 monoclonal antibody (Table 2.5). As this assay was used to inform one of the secondary outcomes of the clinical trial it was formally validated before use as described in section 2.7. For all flow cytometry experiments described, single stain compensation tubes were used to automatically set compensation values prior to running the experimental tubes (Table 2.6) and

events were acquired on a BD LSR II Flow Cytometer (default laser and filter configuration) and analysed using FACS DIVA Software Version 8.0 (BD). Compensation was manually checked off-line for accuracy and corrected where necessary. The photomultiplier tube (PMT) voltages were set at the optimisation stage for each panel and kept constant throughout the experiments with only minor modifications where necessary.

In a proportion of AAV patients and healthy volunteers 100 μL of whole blood was also stained with a different antibody mix (Table 2.7) in order to phenotype CD4+CD28- T-cells for T helper sub-type according to the expression of chemokines CXCR3, CCR6 and CCR4.

In order to directly determine the absolute count of CD4 T-cells, 50 μL of whole blood was aliquoted and stained with a freshly prepared monoclonal antibody mix conjugated to appropriate fluorochromes (Table 2.8) for 30 minutes at 4^{O}C prior to incubating for 15 minutes at room temperature with 450 μL of 1 x Red Blood Cell Lysis Buffer (Biolegend) (10 x Red Blood Cell Lysis Buffer was diluted 1:10 with distilled water). Without washing, 50 μL of counting beads (CytoCount^{TM}, Dako) was added to the sample prior to acquiring events on the flow cytometer.

In order to calculate the cell number of a gated population, the number of events counted for the cells of interest was divided by the number of counting beads counted and multiplied by the concentration of the lot of CytoCount™ beads used in the experiment.

Table 2.5 Antibodies used in the whole blood surface staining assay to determine CD4+CD28- proportions (secondary outcome)

Marker	Fluorochrome	Isotype	Clone	Concentration µL / test	Company
CD3	Brilliant Violet 650	Mouse IgG2a, κ	OKT3	12 µg/mL 2 µL	Biolegend
CD4	Brilliant Violet 605	Mouse IgG2b, κ	OKT4	100 µg/mL 2 µL	Biolegend
CD28	eFluor 450	Mouse IgG1, κ	CD28.2	25 µg/mL 3 µL	eBioscience
Isotype Control	eFluor 450	Mouse IgG1, κ	P3.6.2.8.1	0.2 mg/mL 0.5 µL	eBioscience

Table 2.6 List of flow cytometry antibodies used for single stain compensation

Marker	Channel	Fluorochrome	Isotype	Clone	Concentration µL / test	Company
CD4	FITC	FITC	Mouse, IgG1, κ	RPA-T4	200 µg/mL 3 µL	eBioscience
CD4	PE	PE	Mouse, IgG1, κ	RPA-T4	100 µg/mL 3 µL	eBioscience
CD4	PE-Texas Red	PE-CF594	Mouse, IgG1, κ	RPA-T4	3 µL	BD
CD4	PE-Cy5	PE-Cy5	Mouse, IgG1, κ	RPA-T4	15 µL	BD
CD3	PerCP Cy5.5	PerCP eFluor 710	Mouse, IgG1, κ	SK7	12 µg/mL 3 µL	eBioscience
CD4	PE-Cy7	PE-Cy7	Mouse, IgG1, κ	SK3	12 µg/mL 3 µL	eBioscience
CD4	Pacific Blue	eFluor450	Mouse, IgG1, κ	SK3	25 µg/mL 3 µL	eBioscience
CD4	AmCyan	V500	Mouse, IgG1, κ	RPA-T4	3 µL	BD
CD4	Qdot 605	Brilliant Violet 605	Mouse, IgG2b, κ	OKT4	100 µg/mL 3 µL	Biolegend
CD3	Qdot 655	Brilliant Violet 650	Mouse, IgG2a, κ	OKT3	12 µg/mL 3 µL	Biolegend
CD3	APC	APC	Mouse, IgG1, κ	SK7	25 µg/mL 3 µL	eBioscience
CD4	Alexa Fluor 700	Alexa Fluor 700	Mouse, IgG1, κ	RPA-T4	50 µg/mL 3 µL	eBioscience
CD4	APC-Cy7	APC-Cy7	Mouse, IgG2b, κ	OKT4	200 µg/mL 3 µL	Biolegend

Table 2.7 Antibodies used in a selection of whole blood surface staining experiments to determine T helper and memory sub-type

Marker	Fluorochrome	Isotype	Clone	Concentration µL / test	Company
CD3	Brilliant Violet 650	Mouse IgG2a, κ	OKT3	12 µg/mL 3 µL	Biolegend
CD4	Brilliant Violet 605	Mouse IgG2b, κ	OKT4	100 µg/mL 3 µL	Biolegend
CD28	eFluor 450	Mouse IgG1, κ	CD28.2	25 µg/mL 4 µL	eBioscience
CXCR3	PE-Dazzle 594	Mouse IgG1, κ	G025H7	150 µg/mL 3 µL	Biolegend
CCR4	PE-Cy7	Mouse IgG1, κ	L291H4	200 µg/mL 5 µL	Biolegend
CCR6	PerCP-Cy5.5	Mouse IgG2b, κ	G034E3	100 µg/mL 5 µL	Biolegend

Table 2.8 Antibodies used in the whole blood surface staining assay to directly measure CD4 T-cells

Marker	Fluorochrome	Isotype	Clone	Concentration µL / test	Company
CD3	Brilliant Violet 650	Mouse IgG2a, κ	OKT3	12 µg/mL 3 µL	Biolegend
CD4	Brilliant Violet 605	Mouse IgG2b, κ	OKT4	100 µg/mL 3 µL	Biolegend
CD45	FITC	Mouse IgG1, κ	2D1	200 µg/mL 5 µL	Biolegend

2.6.3 Measuring soluble markers of inflammation and endothelial damage

Soluble markers of inflammation and endothelial damage were measured in plasma. Blood was drawn in an EDTA tube and centrifuged within 30 minutes at 4^{o}C, 3000 rpm for 10 minutes. Plasma was aliquoted in 4 tubes and cryopreserved at -80^{o}C. For the luminex assay, analytes were plexed on a 96-well plate as per the manufacturer's instructions. Each plate contained patient samples and healthy volunteer samples. In the case of clinical trial patient samples, both baseline and 6 month samples from a given patient were run on the same plate in order to avoid inter-assay variability. All samples were run in duplicate. Analytes were purchased from eBioscience and plexed as shown in Table 2.9. Validation details for the analytes used to inform one of the secondary outcomes of the clinical trial arm are described in section 2.7.

The wash buffer and antigen standards were prepared as per the manufacturer's instructions. Next, a 4 fold serial dilution of the antigen standards was prepared. To do this, 200 μL of the reconstituted standard was placed in the first tube and 150 μL of Universal Assay Buffer in the rest of the tubes. 50 μL of standard was transferred to tube 2, mixed, and 50 μL sequentially transferred to the rest of the tubes in order to obtain a 7-point standard curve for the assay panel. The detection antibody mixture was then prepared by combining the detection antibody concentrates of each simplex kit according to the manufacturer's instructions. The 96-well flat bottomed plate was then prepared with the use of a hand held magnetic washer. The antibody magnetic beads for each panel (Table 2.9) were combined, vortexed and an appropriate volume

was added to each well. The beads were allowed 2 minutes to accumulate at the bottom of the plate, then the liquid was removed by rapidly inverting the hand held magnetic plate washer and plate assembly over a waste container, followed by blotting the inverted assembly to remove any residual solution. The antibody magnetic beads were then washed with 150 μL of wash buffer in each well.

Samples were thawed at 37^{o}C and centrifuged at 5000 rpm for 5 minutes in order to remove any debris and fibrin. The supernatant was aspirated and placed in a new tube and the sample vortexed before use. For panels 2, 3 and 4, an appropriate dilution of the samples was made up (Table 2.9). Standards and samples were added onto the plate by adding 25 μL of Universal Assay Buffer into each well followed by 25 μL of the standard or sample as appropriate. The plate was sealed and light protected and shaken at 500 rpm for 2 hours at room temperature. Following this incubation step, the plate was washed twice before adding 25 μL of the working detection antibody mixture into each well. The plate was sealed, light protected and incubated at 500 rpm for 30 minutes at room temperature before being washed twice. Streptavidin – PE was added (50 μl) into each well followed by a 30 minute incubation step at 500 rpm, room temperature and 2 further washes. 120 μL reading buffer was added in each well prior to incubating at 500 rpm for 5 minutes at room temperature and reading the samples on a Biorad Luminex 200 Instrument. Data was analysed using ProcartaPlex Analyst 1.0 Software (eBioscience). Duplicate values were averaged during analysis. Where samples were measured as below the level of detection a value

of 0.01 pg/mL was assigned. For CRP samples measured as above the level of detection a value of 9 x 10^7 pg/mL was assigned.

Table 2.9 List of simplex analytes plexed for the determination of soluble markers of inflammation and endothelial damage in plasma

Analyte	Panel Number	Dilution	Sensitivity	Standard Curve Range
Fractalkine	1	None	0.5 pg/mL	2.08 – 8500 pg/mL
IFN-γ	1	None	0.2 pg/mL	12.21 – 50000 pg/mL
TNF-α	1	None	0.4 pg/mL	8.54 – 35000 pg/mL
IL-2	1	None	0.8 pg/mL	4.88 – 20000 pg/mL
IL-6	1	None	0.4 pg/mL	9.77 – 40000 pg/mL
IL-10	1	None	0.1 pg/mL	2.44 – 10000 pg/mL
IL-12p70	1	None	0.04 pg/mL	6.84 – 28000 pg/mL
IL-17A	1	None	0.1 pg/mL	2.44 – 10000 pg/mL
IP-10	1	None	0.3 pg/mL	1.95 – 8000 pg/mL
MCP-1	1	None	0.6 pg/mL	1.22 – 5000 pg/mL
E-Selectin	1	None	555.3 pg/mL	292.97 – 1200000 pg/mL
P-Selectin	1	None	95.4 pg/mL	1464.84 – 6000000 pg/mL
RANTES	2	1:50	0.2 pg/mL	0.61 – 2500 pg/mL
VCAM	3	1:200	6.7 pg/mL	11.47 – 47000 pg/mL
ICAM	3	1:200	76.3 pg/mL	151.85 – 622000 pg/mL
CRP	4	1:500	0.291 pg/mL	4 – 18000 pg/mL

2.6.4 Peripheral blood mononuclear cell isolation

Blood was drawn in LH tubes and left upright on the bench at room temperature for a maximum of 5 hours before being processed to isolate the peripheral blood mononuclear cell (PBMC) fraction.

Prior to isolating the PBMC fraction, one LH tube from each sample was centrifuged at 584g for 10 minutes in order to isolate plasma. The supernatant was carefully aspirated, aliquoted in 4 tubes in 250 μL aliquots and cryopreserved. Plasma derived from LH blood tubes was used to determine the CMV IgG titre of samples (section 2.6.8). Next, the tube was inverted to re-suspend the whole blood sample and blood was diluted 1:1 with RPMI (Sigma) medium pre-warmed to 37^{o}C. The diluted blood sample was carefully layered over 15 mL Ficoll Paque Plus (GE Healthcare; Endotoxin tested <0.12 EU/mL) in a 50 mL tube. The layered blood was centrifuged at 584 g for 30 minutes at room temperature with the brake off. The lymphocyte layer was then carefully harvested using a Pasteur pipette into a fresh 50 mL tube and warm RPMI medium added to make up the volume to 40 mL before centrifuging at 912 g for 7 minutes. The supernatant was carefully discarded and the cell pellet gently re-suspended. Next, 20 mL RPMI was added, the sample was mixed, and a 20 μl aliquot was placed in a haemocytometer counting slide. The sample was centrifuged again at 584 g for 7 minutes. In order to count the cells the average of 2 4x4 squares on the haemocytometer slide was multiplied by 10,000 and the volume of the sample. The supernatant was then carefully discarded and the cell pellet gently re-suspended to a

concentration of 1 x 10^6 cells / mL with supplemented warm RPMI medium (RPMI, 10% foetal calf serum (FCS; sterile filtered and heat inactivated at 56^{o}C for 30 minutes; Sigma), 1% penicillin / streptomycin (P/S; Gibco)). Cells were then used for culture experiments as appropriate (section 2.6.5).

To cryopreserve, the cells were centrifuged at 584 g for 7 minutes and re-suspended in Freezing Solution A (60% FCS, 40% RPMI) at 20 x 10^6 cells / mL. An equal volume of Freezing Solution B (20% dimethyl sulphoxide (DMSO, Sigma), 80% FCS) was then added in a dropwise fashion with gentle agitation of the tube to achieve a final concentration of 10 x 10^6 cells / mL. Cells were transferred to cryovials and placed in a Mr Frosty (Thermo Scientific) freezing container half filled with 2-propanol (Sigma) for a minimum of 4 hours at -80^{o}C. They were transferred to liquid nitrogen within a maximum of 7 days.

2.6.5 Peripheral blood mononuclear cell stimulation

PBMC were stimulated overnight for 16 hours (+/- 2 hours) at 37^{o}C, 5% CO_2, with CMV lysate (see section 2.6.6). This time period was chosen for practical purposes and based on previous experience of this duration of stimulation with CMV lysate in Professor Moss's lab. For each sample, an unstimulated tube (negative control), an SEB (staphylococcal enterotoxin B; Sigma) stimulated tube (positive control; 200 μg/mL) and 2 CMV lysate stimulated tubes (1:100) (Panel A and Panel B, Table 10)

were set up. Cultures contained 5 x 10^5 – 1 x 10^6 freshly isolated PBMC, monensin (1:1000) to trap cytokines intracellularly, 5 μL CD154 monoclonal antibody (100 μg/mL) conjugated to PE (eBioscience) in order to identify CMV specific cells, and stimulant as appropriate, in a total volume of 500 μL made up with supplemented RPMI. In later experiments the CMV Lysate Panel A tube did not contain CD154 antibody conjugated to PE in order to accommodate staining with an NKG2D monoclonal antibody conjugated to PE as shown in Table 2.10.

The following day cells were washed twice in 2 mL of PBS before staining with the eFluor506 fixable viability dye (eBioscience). The viability dye was used as a 1:10 dilution with PBS at 2 μl per sample and cells were incubated for 30 minutes at 4^{o}C, protected from light, before washing once with PBS and once with MACS buffer. All centrifugation steps were performed at 584 g for 5 minutes at room temperature. Next, the appropriate surface antibody mix was added to the cells (Table 2.10) and cells were gently re-suspended and incubated for 30 minutes at 4^{o}C, protected from light. Following a wash step in 2 mL of MACS buffer, the cells were vortexed to completely re-suspend the pellet and fixed and permeabilised using the FoxP3 one-step fixation and permeabilisation kit (eBioscience). Briefly, cells were incubated in 1 mL of working solution for 30 minutes at 4^{o}C before adding 2 mL of permeabilisation wash buffer and centrifuging for 5 minutes at 584 g. The supernatant was carefully decanted, the cells gently re-suspended and the cells stained with the appropriate intracellular antibody mix (Table 2.10) for 30 minutes at 4^{o}C, protected from light.

Following a final wash in MACS buffer, cells were re-suspended in 200 μl MACS buffer and events were acquired on the flow cytometer. The unstimulated sample was used as a guide to gate for cytokine expression.

Table 2.10 Antibodies used to stain PBMC following overnight stimulation with CMV lysate or SEB

Marker	Fluorochrome	Isotype	Clone	Concentration μL / test	Panel*	Company
Viability	eFluor 506			2 μL	ALL	eBioscience
Surface						
CD3	Brilliant Violet 650	Mouse IgG2a, κ	OKT3	12 μg/mL 3 μL	ALL	Biolegend
CD4	Brilliant Violet 605	Mouse IgG2b, κ	OKT4	100 μg/mL 3 μL	ALL	Biolegend
CD28	eFluor 450	Mouse IgG1, κ	CD28.2	25 μg/mL 4 μL	ALL	eBioscience
NKG2D	PE	Mouse IgG1, κ	1D11	200 μg/mL 5 μL	A	Biolegend
PD-1	APC-Cy7	Mouse IgG1, κ	EH12.2H7	200 μg/mL 5 μL	B	Biolegend
TIM-3	PE-Cy7	Mouse IgG1, κ	F38-2E2	200 μg/mL 5 μL	B	Biolegend
LAG-3	Fluorescein	Goat IgG (polyclonal)	Leu23-Leu450	10 μL	B	R&D
Intracellular						
IFN-γ	PE-CF594	Mouse IgG1, κ	B27	3 μL	ALL	BD
TNF-α	Alexa Fluor 700	Mouse IgG1, κ	MAb11	50 μg/mL 3 μL	ALL	eBioscience
IL-2	PerCP eFLuor 710	Mouse IgG2a, κ	MQ1-17H12	12 μg/mL 3 μL	ALL	eBioscience
IL-10	PE-Cy7	Rat IgG1, κ	JES3-9D7	20 μg/mL 5 μL	U, SEB, A	Biolegend
IL-5	APC	Rat IgG1, κ	TRFK5	0.2 mg/mL 5 μL	U, SEB, A	Biolegend
T-bet	FITC	Mouse IgG1, κ	4B10	0.5 mg/mL 2 μL	U, SEB, A	Biolegend
BLIMP-1	APC	Mouse IgG1, κ	646702	10 μL	B	R&D
CTLA-4	PE-Cy5	Mouse IgG2a, κ	BNI3	20 μL	B	BD

* U: unstimulated; SEB: SEB stimulated; A: CMV Lysate Panel A; B: CMV Lysate Panel B

2.6.6 CMV lysate production

To prepare CMV lysate, foetal human foreskin fibroblasts (HFFF) were cultured in 20 mL supplemented Dulbecco's modified eagle medium (DMEM; Sigma) (10% FCS, 1% P/S) in 150 cm^2 tissue culture flasks (37°C, 5% CO_2) and allowed to become 70-80% confluent over a period of 5 days.

The cells were then split into three 150 cm^2 flasks and incubated for a further 5 days as above by aspirating the culture medium, gently washing with 8 mL PBS twice, treating with trypsin to detach the HFFF (2 mL TryPLE Express (Sigma) added and cells incubated at 37°C, 5% CO_2 for 5 minutes)), adding culture medium and splitting. The process was repeated until 15 flasks with cultured HFFF were generated.

Ten of the 15 flasks were then infected with CMV AD169 strain (stock solution - titre 3 x 10^7 PFu / mL) at a multiplicity of infection (MOI) of 0.05 (50 μL of CMV AD169 stock solution was mixed with 50 mL culture medium and 10 mL was added to each flask having aspirated the existing culture medium first) whilst 5 flasks were kept to produce mock lysate. Flasks were incubated at 37°C, 5% CO_2 for 4 hours with gentle rocking every 4 hours after which time the supernatant was aspirated and flasks were replenished with 20 mL of fresh culture medium.

Once the HFFF displayed cytopathic changes under the microscope (5-10 days) the supernatant was harvested 3 times (each time 3 days apart) by carefully removing the supernatant and replenishing with fresh culture medium. The harvested supernatant was centrifuged at 2000 rpm for 10 minutes and the ensuing supernatant combined into sterile Sorvall pots and frozen at -80°C whilst the pellets were combined and also frozen at -80°C.

On the final harvest the HFFF cells were scraped off and centrifuged together with the supernatant at 2000 rpm for 10 minutes. The supernatant was then added to the thawed Sorvall pots and the cell pellets combined with the thawed pellets from the previous harvests. The combined infected cell pellet preparation was centrifuged at 2000 rpm for 10 minutes, re-suspended to a final volume of 1 mL with RPMI and freeze-thawed 5 times in liquid nitrogen. Meanwhile the Sorvall pots were centrifuged at 12000 rpm for 2 hours at room temperature. The supernatant from the Sorvall pots was appropriately discarded and the pellets resuspended at a final volume of 6 mL RPMI and combined with the freeze-thawed infected cell pellet. The final infected virus pellet was sonicated 5 times for 20 seconds at a time, aliquoted and stored at -80°C.

For the mock lysate, following collection of the supernatant and cell scrapings, the suspension was centrifuged at 2000 rpm for 10 minutes, the supernatant appropriately discarded and the uninfected cell pellet made up to a total volume of 3 mL, freeze-

thawed, sonicated, aliquoted and stored as above. The same batch of CMV and mock lysate were used throughout all experiments in the study in order to avoid variability between different lysate batches.

The volume of CMV lysate required for overnight stimulation was titrated in initial experiments (Figure 2.5) and 5 μl of CMV lysate (1:100) used in all subsequent experiments. The specificity of the CMV lysate was also tested in initial experiments that showed IFN-γ production by CD4 cells only in CMV seropositive individuals in response to CMV lysate and no IFN-γ production when cells from a CMV seropositive individual were stimulated with mock lysate (Figure 2.6).

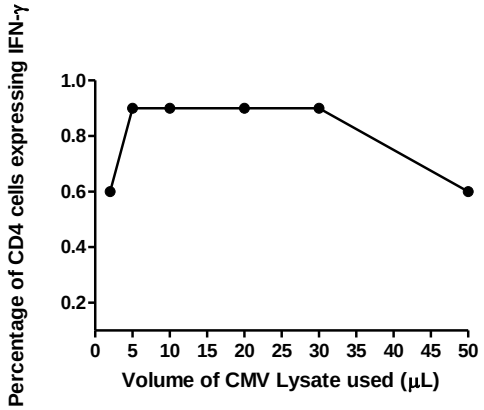

Figure 2.5 CMV lysate titration

Initial titrating experiments showed that 5 μL of CMV lysate in culture used at 1:100 produced a good response

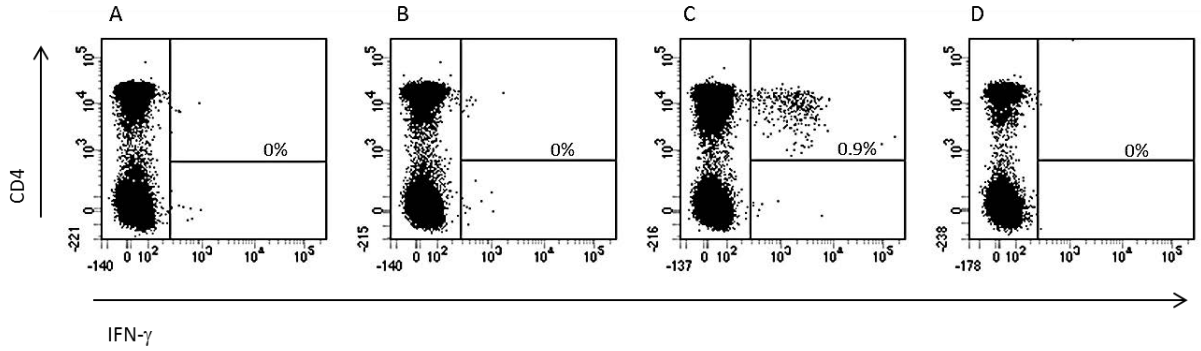

Figure 2.6 Flow cytometry data showing specificity of CMV lysate stimulation
A, unstimulated PBMC from a CMV seropositive donor. B, mock lysate stimulated PBMC from a CMV seropositive donor. C, CMV lysate stimulated PBMC from a seropositive donor. D, CMV lysate stimulated PBMC from a seronegative donor.

2.6.7 CD4+CD28null percentage tracking experiments

The CD4+CD28- T-cell percentage over a 12 month period was tracked for a subset of patients that took part as controls in the clinical trial and had significant CMV reactivation documented in order to determine whether CMV reactivation led to a change in the CD4+CD28null T-cell percentage. For these experiments PBMC were thawed at 37^{o}C before adding warm supplemented RPMI medium to the cryovial containing the PBMC in dropwise fashion. The PBMC were gently mixed and slowly added to a 50 mL tube containing 20 mL of warm supplemented RPMI. The cells were then centrifuged for 7 minutes at 584 g room temperature, the supernatant carefully decanted and the pellet gently re-suspended with 20 mL warm supplemented RPMI medium before counting on a haemocytometer slide (section 2.6.4). Following

another centrifugation step, cells were re-suspended at 1 x 10^6 cells / mL with warm supplemented RPMI medium.

Cells from all time points from one patient were thawed and batch analysed in a single experiment in order to avoid inter-assay variability. For each time point, 5 x 10^5 – 1 x 10^6 cells, depending on cell numbers, were first washed in PBS and stained with viability dye as described in section 2.6.5 before staining with the surface marker panel detailed in Table 2.11, washing in MACS buffer, and acquiring on the flow cytometer.

Table 2.11 Antibodies used to stain cells in CD4+CD28null T-cell tracking experiments

Marker	Fluorochrome	Isotype	Clone	Concentration μL / test	Company
Viability	eFluor 506			2 μL	eBioscience
CD3	Brilliant Violet 650	Mouse IgG2a, κ	OKT3	12 μg/mL 3 μL	Biolegend
CD4	Brilliant Violet 605	Mouse IgG2b, κ	OKT4	100 μg/mL 3 μL	Biolegend
CD28	eFluor 450	Mouse IgG1, κ	CD28.2	25 μg/mL 4 μL	eBioscience

2.6.8 Estimation of CMV IgG titre

Plasma sample collected in LH tubes (section 2.6.4) was used to determine CMV IgG titres across the clinical trial period. A CMV IgG ELISA assay developed at the University of Birmingham by Professor Paul Moss's group was used. All time points from a given patient were run on the same plate in order to avoid inter-assay variability. A sample from a known CMV seropositive donor (positive control) and a known CMV seronegative donor (negative control) for which multiple aliquots were frozen was run with every single plate in order to document assay precision and reproducibility. All samples were run in duplicate in CMV lysate coated wells, as well as mock lysate coated wells, and the average absorbance value seen in the mock lysate coated wells (background non-specific binding) was subtracted from the average absorbance value in the CMV lysate coated plate. Clinical trial samples assayed for CMV IgG titre were processed with the help of Dr Michael Sagmeister as part of a mini-project during Dr Sagmeister's renal medicine academic block that was jointly supervised by the author of this thesis and Professor Lorraine Harper.

Briefly, a MaxiSorp flat-bottom 96 well plate (Nunc) was coated with inactivated CMV lysate and mock lysate. The same batch of lysate was used for all experiments in order to minimise inter-assay variability. Coating buffer was made up by dissolving one carbonate-bicarbonate capsule (Sigma) in 25 mL of distilled water. Lysate was used in a 1:4000 dilution and wells were coated with 50 μL of CMV lysate or mock lysate as

shown in Figure 2.7 and incubated overnight at 4^{o}C, protected from light, for 16 hours (+/- 1 hour).

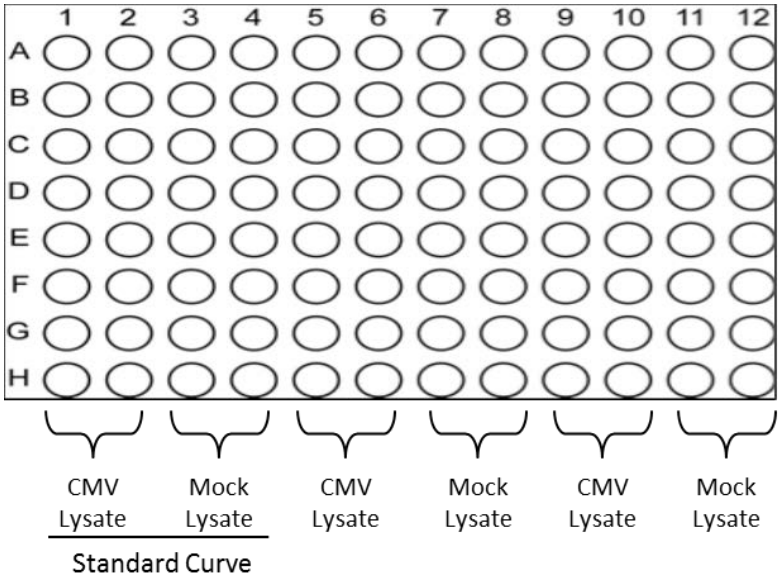

Figure 2.7 Determination of CMV IgG titre

To determine CMV IgG titre a 96 well flat bottomed plate was coated with CMV and mock lysate as shown and incubated overnight.

The following morning the plate was washed 3 times with 200 μL per well of wash buffer (PBS, 0.05% Tween 20; Sigma). The samples and standards were prepared by thawing at 37^{o}C and centrifuging at 5000 rpm for 5 minutes in a microcentrifuge to pellet debris and fibrin in order to avoid background non-specific binding. The

supernatant was aliquoted in a fresh tube and vortexed before use. Standards were made up from a 1:1:1 mix of 3 donors that remained constant throughout all experiments. A 3-fold dilution of the standard was produced by adding 18 μL of the standard to 72 μL of dilution buffer (wash buffer, 1% bovine serum albumin (BSA); Sigma), mixing, transferring 30 μL to the next well that contained 60 μL dilution buffer and repeating this process to achieve a 7 point standard curve preparation in both the CMV lysate and mock lysate coated wells. The last well was left blank.

Sample, positive and negative controls were used at a 1:3000 dilution in dilution buffer. Once standards, samples and controls were added to the wells they were incubated for 1 hour at room temperature before washing 3 times and adding 100 μL per well of goat anti-human IgG conjugated to horse radish peroxidase (HRP) (Southern Biotech) at a 1:8000 dilution. Following incubation for 1 hour at room temperature, the plate was washed 3 times before adding 100 μL per well of the detection agent (Tetramethyl Benzidine (TMB) One Component HRP Microwell Substrate; Tebu-Bio) and incubating for 15 minutes at room temperature in the dark. The reaction was stopped by adding 100 μL per well of a 1M solution of hydrochloric acid and the plate read at 450 nm absorbance.

To calculate the CMV IgG titre the average absorbance values of the mock lysate duplicates were subtracted from the average absorbance values of the CMV lysate

duplicates for each sample and for the standard curve. The values were then log transformed and a non-linear regression sigmoidal dose response curve was fitted (Figure 2.8) in order to interpolate the sample values based on the standard curve.

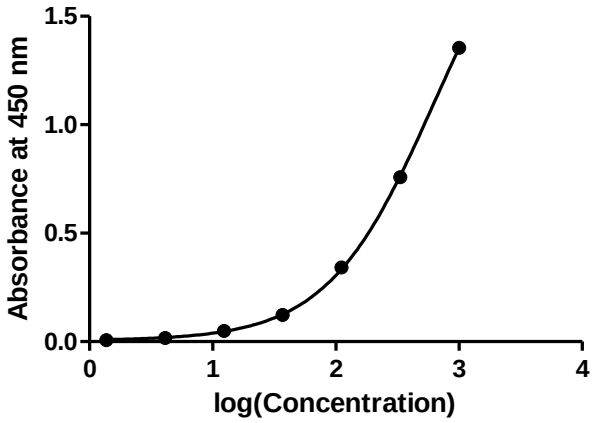

Figure 2.8 CMV IgG ELISA standard curve

Example standard curve for the CMV IgG titre ELISA assay following log transformation of the absorbance values for the 7-point standard curve samples.

The interpolated values were then exponentially transformed to yield the CMV IgG titre. A value of greater than 10 units indicated a positive result in terms of CMV serological status based on previous validation studies by Professor Paul Moss's group.

2.7 Formal Assay Validation Analyses

The assays used to inform the primary and secondary outcomes of the clinical trial arm of the study were formally validated as per MHRA requirements prior to the commencement of the study. The qPCR assay used to determine the primary outcome

has been formally validated for clinical use by UHB Virology Laboratories and was therefore not further validated for the purposes of this clinical trial. The whole blood surface staining assay used to determine the proportion of CD3+CD4+CD28- cells and the Luminex assay used to determine plasma concentration of soluble markers of inflammation were formally validated. Excerpts of the validation analyses are presented here whilst the full validation plans and analyses can be found in Appendix 7.

2.7.1 Surface Staining of CD3 CD4 CD28 in Whole Blood – Validation Analysis Part 1

2.7.1.1 Objective

The aim of this validation study was to establish that the performance characteristics of the assay that would be used to surface stain lymphocytes in whole blood for CD3, CD4 and CD28, met the analytical requirements of the CANVAS trial.

2.7.1.2 Validation Parameters

In order to examine whether the period of time blood is left at room temperature prior to processing affects the proportion of CD3+CD4+CD28- cells within the CD4 compartment, blood was drawn from healthy volunteers and left at room temperature for 1 hour, 3 hours and 5 hours before being stained (flow cytometry panel as detailed

in Table 2.5) and analysed as previously described. This experiment was repeated three times (Figure 2.9). The one-sample Wilcoxon signed rank test was used to determine whether the median CD3+CD4+CD28- proportion at 3 hours and 5 hours was statistically different to that at 1 hour. In order to test intra-assay variability all experiments were carried out in duplicate and the coefficient of variation was calculated.

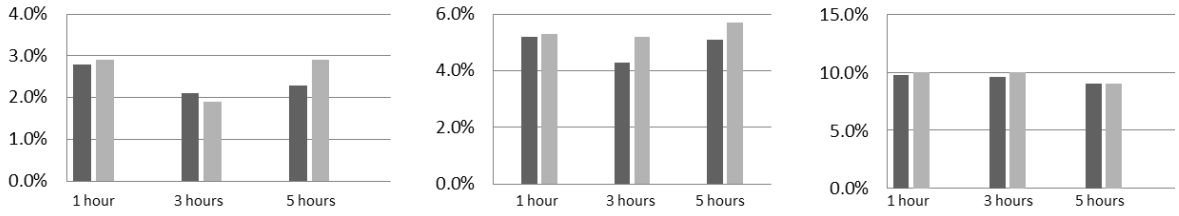

Figure 2.9 CD3 CD4 CD28 staining validation

Effect of time elapsed from venepuncture to staining on the CD3+CD4+CD28- proportion within the CD4 compartment (y-axes). Three separate experiments (different donors) are shown where staining was performed in duplicate after resting whole blood at room temperature for 1, 3 and 5 hours. Coefficients of variation were < 20% for all duplicate runs. There was no statistically significant difference between the mean CD3+CD4+CD28- % at 3 hours and 5 hours versus 1 hour in all experiments.

2.7.1.3 Acceptance Criteria

There was no statistically significant difference between the mean CD3+CD4+CD28- % at 3 hours and 5 hours versus 1 hour at all experiments (also see full Validation Analysis Report in Appendix 7). Therefore it was decided that following collection, blood samples could be left at room temperature for up to a maximum of 5 hours before processing.

The coefficient of variation was acceptable throughout the validation analysis study. However it was decided that a positive control with a pre-defined acceptance range of CD3+CD4+CD28+ cells was necessary in order to properly validate the various components of the assay in terms of intra-assay and inter-assay variability and also allow running of an aliquot of the positive control with each experimental run for quality control purposes.

2.7.2 Surface Staining of CD3 CD4 CD28 in Whole Blood – Validation Analysis Part 2

2.7.2.1 Precision and Reproducibility

The precision and reproducibility of the whole blood staining assay was further validated using a commercially available positive control (Cytofix CD4 Normal Range Positive Control; Cytomark, Caltag MedSystems). Cytofix CD4 controls are a stabilised

preparation of whole human blood for use as a positive control when evaluating CD4 enumeration techniques. They provide verification of monoclonal antibody reagents and red blood cell lysis as well as validation of instrument performance and operator technique. Each lot of Cytofix CD4 positive control is provided with a quality control statement inclusive of the expected range of CD3+CD4+ lymphocytes within each lot.

Positive controls from the first lot purchased were initially assayed in triplicate on five separate experiments as previously described (Section 2.6.2; flow cytometry panel as detailed in Table 2.5) in order to define intra-assay (Figure 2.10) and inter-assay variability as well as acceptance criteria for the assay based on the positive control CD3+CD4+CD28+ range (Table 2.12). The acceptance range for each marker was calculated as the mean value across the five triplicate runs +/- 2 standard deviations. As the coefficient of variation in terms of intra-assay variability was very low (Figure 2.10), it was decided that experimental samples and controls could be run singly for the purposes of the clinical trial.

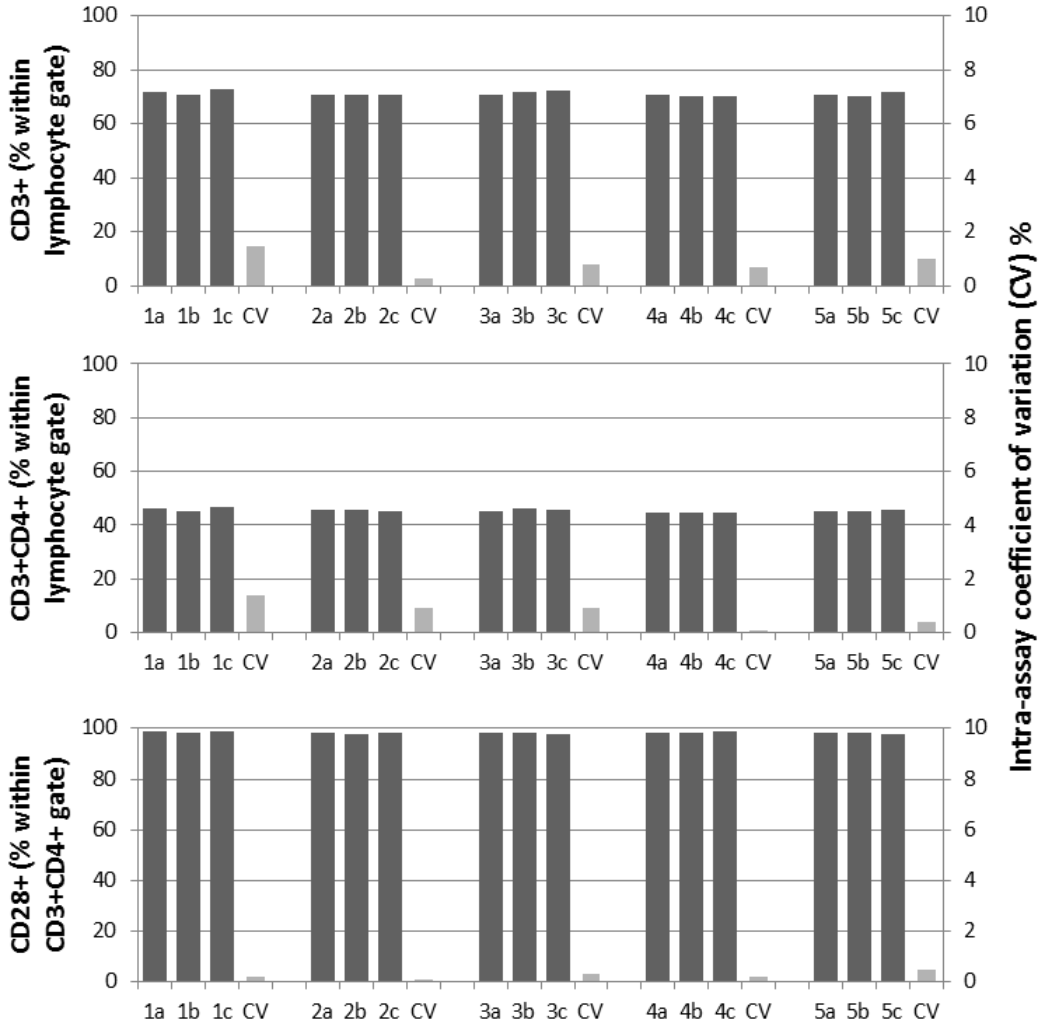

Figure 2.10 CD3 CD4 CD28 staining validation

Cytofix CD4 Positive Control was stained in triplicate (a, b, c) in 5 separate experiments (1-5). Top panel shows the percentage of CD3+ cells, middle panel the percentage of CD3+CD4+ cells and bottom panel the percentage of CD3+CD4+CD28+ cells. The intra-assay coefficient of variation (%) is shown in the light grey bars (right sided y-axes).

An aliquot of the Cytofix CD4 positive control was planned to be run alongside each experimental run to ensure that the relevant cell populations within the positive control fell within the validated accepted ranges therefore providing on-going quality assurance of the precision of this assay.

Initial experiments showed that the CD28 expression in CD4 cells of the Cytofix CD4 positive control altered significantly with time. This made the setting of an acceptance range for CD28 expression in the positive control not possible as this value constantly changed during the 1-month shelf life of the product after opening the vial (Appendix 7). Furthermore it was noted that most of the change occurred by 1-2 weeks after the preparation of a new lot of the Cytofix CD4 positive control. It was therefore decided that an acceptance range would be set for CD3 and CD4 expression in the Cytofix CD4 positive control but not for CD28. For every new lot of Cytofix CD4 control used, the expected CD3+CD4+ range for the purposes of its use in the secondary outcome assay acceptance criteria was re-validated by running a sample from the new lot in triplicate. In order to maintain quality assurance of CD28 staining in the experimental samples, an FMO control was assayed with every experimental sample in order to guide CD28 gating.

Table 2.12 Inter-assay variability and initial acceptance ranges for positive control used in the whole blood surface staining assay

	Inter-assay CV (%)	Mean (%)	Acceptance Range
CD3+ within lymphocyte gate	0.9	71.2	70.0 – 72.4
CD3+CD4+ within lymphocyte gate	1.2	45.5	44.4 – 46.6
CD28+ within CD3+CD4+ gate	0.2	98.2	97.7 – 98.7

2.7.3 Measurement of soluble markers of inflammation – Validation Analysis

2.7.3.1 Objective

The aim of this validation study was to establish that the performance characteristics of the assay that was used to determine the plasma concentration of IL-2, TNF-α, IFN-γ, IL-6, IL-10, IL-17 and highly sensitive C-reactive protein (hsCRP) at baseline and at 6 months (secondary outcome), met the analytical requirements of the CANVAS trial.

2.7.3.2 Precision and Reproducibility

The Luminex assay permitted the plasma concentration of IL-2, TNF-α, IFN-γ, IL-6, IL-10, IL-17 and hsCRP to be measured in a multiplex immunoassay. The Luminex

multiplex immunoassay has excellent precision and reproducibility as evidenced by low coefficient of variation (CV) values with regards to intra-assay and inter-assay variability (Table 2.13). This was a commercial assay that has been rigorously validated by the company. Hence no further validation was planned.

Table 2.13 Coefficient of variation (CV) values for intra-assay and inter-assay variability for analytes used to inform the clinical trial secondary outcome

Analyte	**Intra-Assay Variability CV (%)**	**Inter-Assay Variability CV (%)**
IFN-γ	3.6	5.5
TNF-α	7.2	6.1
IL-2	6.2	5.1
IL-6	6.2	4.5
IL-10	3.1	2.3
IL-17	6.5	6.0
hsCRP	6.6	9.6

Source: eBioscience

2.8 Data Collection and Quality Control

Sample collection and processing was evidenced on appropriate worksheets (see Blood Sample Receipt and Processing Record in Laboratory Manual; Appendix 6). Similarly, experimental detail for assays employed within the clinical trial was documented in real time (see Whole Blood / Positive Control Surface Staining Work Instruction in Laboratory Manual; Appendix 6). These records are filed in the CANVAS Study Laboratory Folder and archived at the University of Birmingham. Detailed work instructions for laboratory assays can also be found in the CANVAS Laboratory Manual (Appendix 6).

Source data relating to clinical trial participants was documented in the Case Report Form (Appendix 8). In order to maintain data integrity a standard operating procedure was designed around maintaining quality control of data input (Appendix 5). To that effect, prior to any analysis being undertaken, the accuracy of data transcription was checked for data relating to primary and secondary outcomes of the clinical trial. Serious adverse events (SAE) were documented (Appendix 2; SAE form in Protocol) and reported to the sponsor yearly in the Development Safety Update Report (DSUR) (Appendix 9).

2.9 Data Presentation and Statistical Analysis

For the purposes of statistical analysis, SPSS Statistics Version 21 (IBM) and Prism Version 5 and 6 (GraphPad) were used. Data was assessed for normality using the D'Agostino & Pearson omnibus normality test and aided by visual inspection of graphical representation (histogram and Q-Q plots) of the data and skewness values in borderline cases. Non-parametric data was transformed when necessary using square root or logarithmic transformations and re-tested for normality as above. Parametric data is reported as mean with standard deviation (SD) and non-parametric data as median and interquartile range (IQR). Non parametric data is graphically depicted in the form of Tukey boxplots or scatter dot plots with median and interquartile range shown. Parametric data is depicted with mean and standard error of the mean shown.

All statistical analyses were two-tailed. Unpaired parametric and non-parametric data were analysed using the student's t test (with Welch's correction for unequal variances if appropriate) or the Mann Whitney U test respectively. Paired data was analysed using the paired t test or the Wilcoxon-matched pairs signed rank test for parametric and non-parametric data respectively. Categorical data was analysed using the chi square test or Fisher's exact test where appropriate. Correlations were assessed with Spearman's rank test for non-parametric data and Pearson's correlation for parametric variables. A p value < 0.05 was considered statistically significant for all analyses.

2.9.1 Clinical Trial data presentation and statistical analysis

All clinical trial analyses were carried out following the intention to treat principle as pre-specified in the published clinical trial protocol [325] unless explicitly stated within the text. A secondary per protocol analysis that excluded patients randomised to the treatment arm that stopped the drug within 1 month from starting the trial was additionally performed on the clinical trial primary outcome data. The primary analysis on the primary outcome was carried out utilising the UHB virology laboratory cut-off of >200 CMV viral DNA copies / mL to define CMV reactivation. Additional secondary analyses were carried using a >20 CMV viral DNA copies / mL threshold. Kaplan-Meier curves were drawn to analyse the primary outcome and the curves were compared using the Gehan-Breslow-Wilcoxon test. As the primary and secondary analyses for the primary outcome only considered time to 1st episode of CMV reactivation, a sensitivity analysis was also carried out for the primary outcome to consider multiple episodes of CMV reactivation in the same patient.

The secondary, tertiary and exploratory outcomes of the clinical trial were comprised of paired data (e.g. CD4+CD28null % at month 0 and month 6). For such paired data the paired t test was utilised. As the ratios of paired values were expected to be more consistent than the differences between paired values, paired ratio t tests were employed for such outcome data. In order to achieve this, data was log transformed and a two-tailed t test (or Wilcoxon matched-pairs signed rank test for non-parametric data) was carried out on the transformed values. The antilog of the results was then

taken to derive the geometric mean of the ratios with 95% confidence intervals. This was performed separately for the treatment and control groups.

Where outcome variables were assayed on more than two time-points, repeated measures ANOVA was carried out. To account for multiple comparisons between the different time points examined in the tertiary outcome, post hoc analysis was carried out using Dunnett's multiple comparisons test. For the change in CMV IgG titre exploratory analysis, a post hoc analysis for linear trend was carried out to determine whether there was a change in titre over the study period.

CHAPTER 3

THE PHENOTYPE OF CD4+CD28null T-CELLS IN ANCA ASSOCIATED VASCULITIS

Chapter 3 The Phenotype of CD4+CD28null T-cells in AAV

3.1 Introduction

Increased expansions of CD4+CD28null T-cells have been described in numerous inflammatory disorders including ANCA associated vasculitis (AAV) as well as in cardiovascular disease and in association with advancing age [112, 153, 159, 175, 188, 212, 221]. CD4+CD28null T-cells are believed to be a pro-inflammatory subset [184]. Although significant expansions of CD4+CD28null T-cells are only present in CMV seropositive individuals [112, 168, 224], the origin of this subset continues to be debated in the literature [171, 184, 200]. Furthermore, there is a lack of consensus as to whether functional exhaustion exists within this subset and whether this may predispose to increased and more frequent cycles of subclinical CMV reactivation thereby resulting in increased expansion of CD4+CD28null T-cells. Finally, although CD4+CD28null T-cells have been linked to increased mortality, reduced renal function and increased risk of infection in AAV, their phenotype in AAV CMV seropositive patients has not been adequately defined.

The work undertaken in this chapter aimed to:

- Determine the cytokine production of CD4+CD28null T-cells following stimulation with CMV lysate in AAV and compare this to their CD4+CD28+ counterparts and age matched healthy volunteers
- Identify the factors associated with CD4+CD28null T-cell expansion in AAV

- Determine the inhibitory receptor expression profile of CMV specific CD4+CD28null T-cells in AAV and explore how this relates to their ability to produce multiple cytokines
- Characterise CD4+CD28null T-cells in AAV in terms of their T helper subtype and compare this to age matched healthy volunteers

3.2 Cohort Characteristics

In order to phenotype CD4+CD28null T-cells in AAV, 53 patients with stable disease and evidence of past infection with CMV (as evidenced by the presence of anti-CMV IgG antibodies in serum) were recruited from the University Hospital Birmingham (UHB) vasculitis clinic for a single visit in this cross-sectional part of the study. Thirty-eight of those patients were also consented to participate in the clinical trial part of the thesis (Chapter 5). In order to preserve homogeneity, the remaining 15 patients recruited purely into the cross-sectional element of the study fulfilled the same eligibility criteria as the clinical trial participants (Chapter 2, Table 2.3).

Thirty CMV seropositive healthy volunteers (HV) were recruited in order to compare and contrast the phenotype of CD4+CD28null T-cells in AAV and in the absence of immunosuppression or an inflammatory condition.

Table 3.1 describes the characteristics of the AAV patients and HV recruited into the cross sectional part of the study. There were no statistically significant differences between AAV patients and HV in age or gender. AAV patients had a median duration of disease of 71.5 months, mean eGFR of 53 mL / min / 1.73 m^2 and low levels of proteinuria. The majority of AAV patients were on immunosuppressant medication with a combination of prednisolone and azathioprine or mycophenolate mofetil.

Table 3.1 Cohort characteristics for cross-sectional element of study

	AAV (n=53)	**HV (n=30)**	**p value**
Age (years)	69.0 [62.8 - 75.3]	70.5 [66.8 - 74.0]	0.557
Gender (M:F)	35:18	14:16	0.085
ANCA specificity (PR3:MPO)	27:10	--	--
AAV disease chronicity (months)	71.5 [38.0 - 144.0]	--	--
Renal function eGFR (mL/min/1.73m^2)	53 [21]	--	--
Urine albumin creatinine ratio (uACR)	4.4 [1.4 - 9.9]	--	--
Steroids [n (%)]	39 (73.6)	--	--
MMF [n (%)]	14 (26.4)	--	--
Azathioprine [n (%)]	19 (35.8)	--	--
No treatment [n (%)]	4 (7.5)	--	--

Data is displayed as median [interquartile range] apart from renal function that is displayed as mean [standard deviation].

3.3 CD4+CD28null T-cell proportion and absolute count

In order to assess the proportion of CD4+CD28null T-cells within the CD4 compartment, whole blood was stained with CD3, CD4 and CD28 antibodies as described in Chapter 2. The gating strategy is shown in Figure 3.1. A fluorescence-minus-one (FMO) control was used for all experiments in order to assist with gating. CD3+CD4- T-cells were assessed as a surrogate marker of CD8+ T-cells and shall be referred to as CD8 for the remainder of the thesis.

3.3.1 AAV patients display a higher proportion of CD4+CD28null T-cells compared to HV

AAV patients displayed a significantly higher percentage of CD4+CD28null T-cells compared to HV (median 11.3 [IQR 3.7 - 19.7] vs. 6.7 [2.4 - 8.8]; p=0.022) (Figure 3.1). However there was no difference in CD4+CD28null absolute cell count between AAV and HV (Figure 3.2, Panel D). There was no statistically significant difference in the percentage of CD8CD28null T-cells between AAV patients and HV (60.0 [41.0 - 73.6] vs. 50.7 [40.5 - 62.9]; p=0.121).

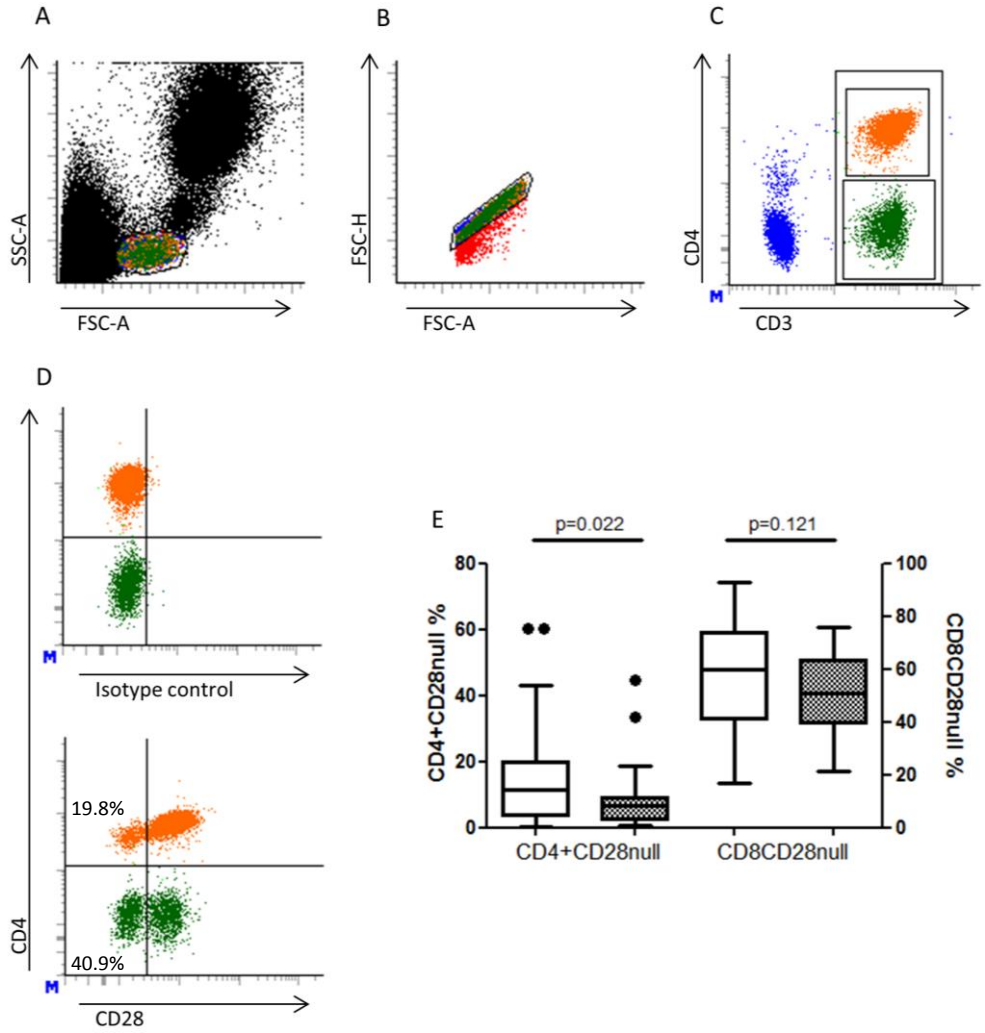

Figure 3.1 CD4+CD28null and CD8CD28null T-cell expression in AAV and HV

Whole blood was stained with CD3, CD4 and CD28 or isotype control monoclonal antibodies and CD4+CD28null and CD8CD28null T-cells were identified as shown here in a representative patient sample: forward and side scatter was used to gate on lymphocytes (panel A); single cells were gated based on forward scatter height and area parameters (panel B); CD3+CD4+ and CD3+CD4- (CD8) cells were next identified (panel C); an FMO control was used to assist with CD28 gating as shown in panel D where the top panel has been stained with an isotype control and the bottom panel with CD28. Panel E: AAV patients (n=53) (white boxes) have a statistically significant higher proportion of CD4+CD28null (left y axis) but not CD8CD28null T-cells (right y axis) compared to HV (n=30) (shaded boxes) (Mann-Whitney U test). Median and Tukey boxplots are shown.

3.3.2 AAV patients have an inverted CD4:CD8 ratio compared to HV

CD4 and CD8 T-cells were enumerated in whole blood (Figure 3.2). AAV patients had a significantly lower CD4 count compared to HV, as previously reported [336], in keeping with an immunosuppressed state (430.4 cells / μL [274.6 - 680.9] vs. 774.9 [590.4 - 1126.3]; $p<0.001$). Although AAV patients also displayed a lower CD8 count compared to HV (292.8 cells / μL [179.7 - 548.1] vs. 426.8 [298.8 - 599.7]; $p=0.019$), the CD4:CD8 ratio in AAV patients was inverted and significantly lower in AAV compared to HV (1.3 [0.9 - 1.7] vs. 1.8 [1.6 - 2.0]; $p=0.008$). The percentage of CD4+CD28null T-cells was negatively correlated to the CD4:CD8 ratio (Figure 3.2), suggesting that the CD4:CD8 ratio, a marker of the immune risk profile [288], which has been associated with CMV past infection and frailty in the elderly [294], is adversely affected by the size of the CD4+CD28null T-cell expansion.

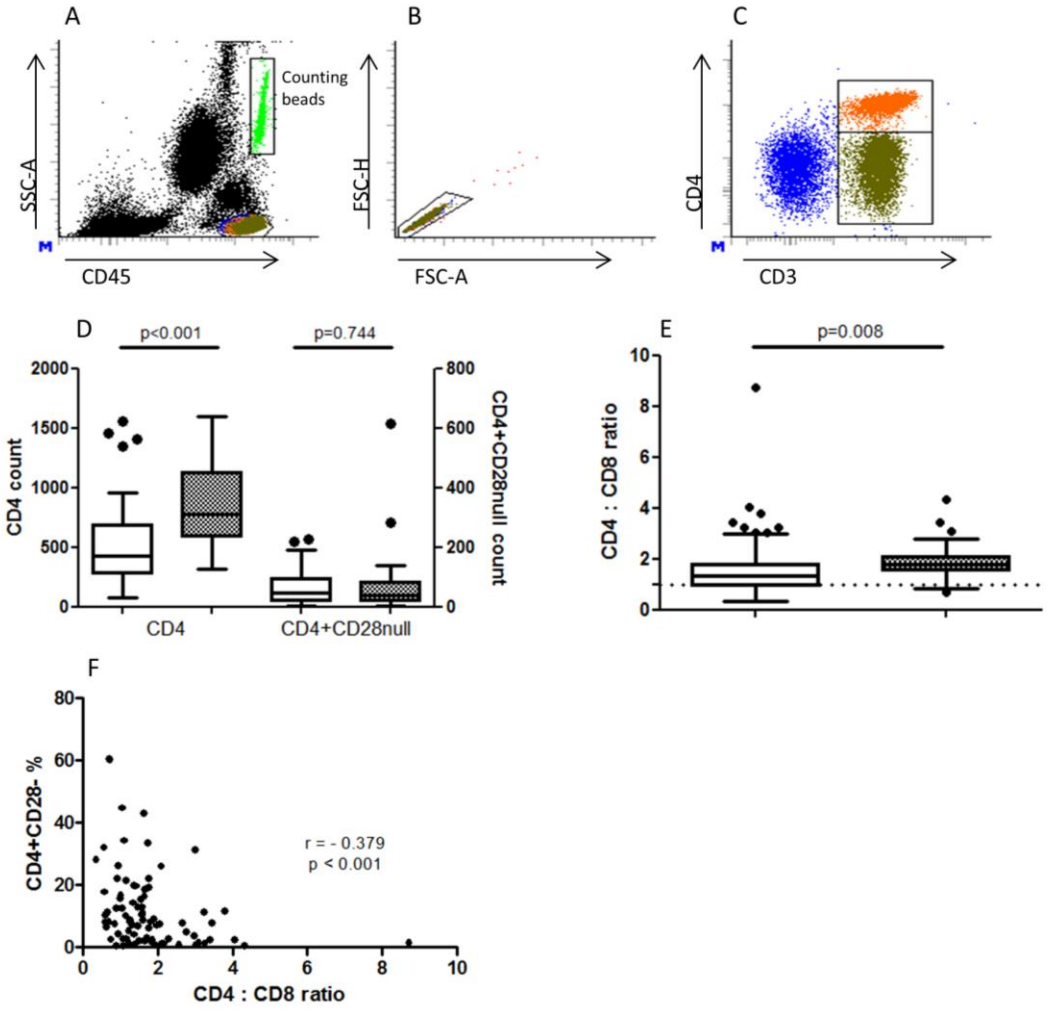

Figure 3.2 CD4 count and CD4:CD8 ratio in AAV patients versus HV

Whole blood was stained with CD45, CD3 and CD4 monoclonal antibodies prior to the addition of counting beads. Panels A, B and C display the gating strategy in a representative patient sample. Panel D shows that AAV patients (n=53) (white boxes) had a statistically significant lower CD4 count compared to HV (n=30) (shaded boxes) (left y axis). There was no difference in CD4+CD28null absolute cell count (right y axis). In Panel E, the CD4:CD8 ratio in AAV patients (white boxes) and HV (shaded boxes) is displayed. The dotted line represents a CD4:CD8 ratio of 1. In Panel F, the CD4+CD28null T-cell proportion is negatively correlated to the CD4:CD8 ratio (n=83).

3.4 CD4+CD28null T-cell phenotype and relationship to the CD4 CMV response

In order to identify CMV responsive CD4+ T-cells and determine the cytokine secreting potential of CD4+CD28null T-cells, peripheral blood mononuclear cells (PBMC) were stimulated with CMV lysate in an overnight (16 hour) culture in the presence of monensin as detailed in Chapter 2.

CD4+CD28null T-cells were identified as CMV responsive by the presence of IFN-γ staining following CMV lysate stimulation. The specificity of this technique for CMV has been discussed in Section 2.6.6 (Figure 2.6).

A significantly greater percentage of CD4+CD28null than CD4+CD28+ T-cells responded to CMV lysate (Figure 3.3). There was no statistically significant difference between AAV and HV in the percentage of CD4+CD28null T-cells that responded to CMV lysate (AAV: 21.2 [IQR 8.3 - 35.9]; HV: 15.2 [6.0 - 42.6]; p=0.776). The proportion of CD4+CD28null T-cells that were able to respond to CMV by IFN-γ production increased as the size of the CD4+CD28null T-cell compartment expanded indicating that individuals with greater expansions of CD4+CD28null have a greater proportion of CD4+CD28null T-cells able to respond to CMV and suggesting that CD4+CD28null T-cell expansion is not associated with loss of IFN-γ production (Figure 3.3, Panels C and D).

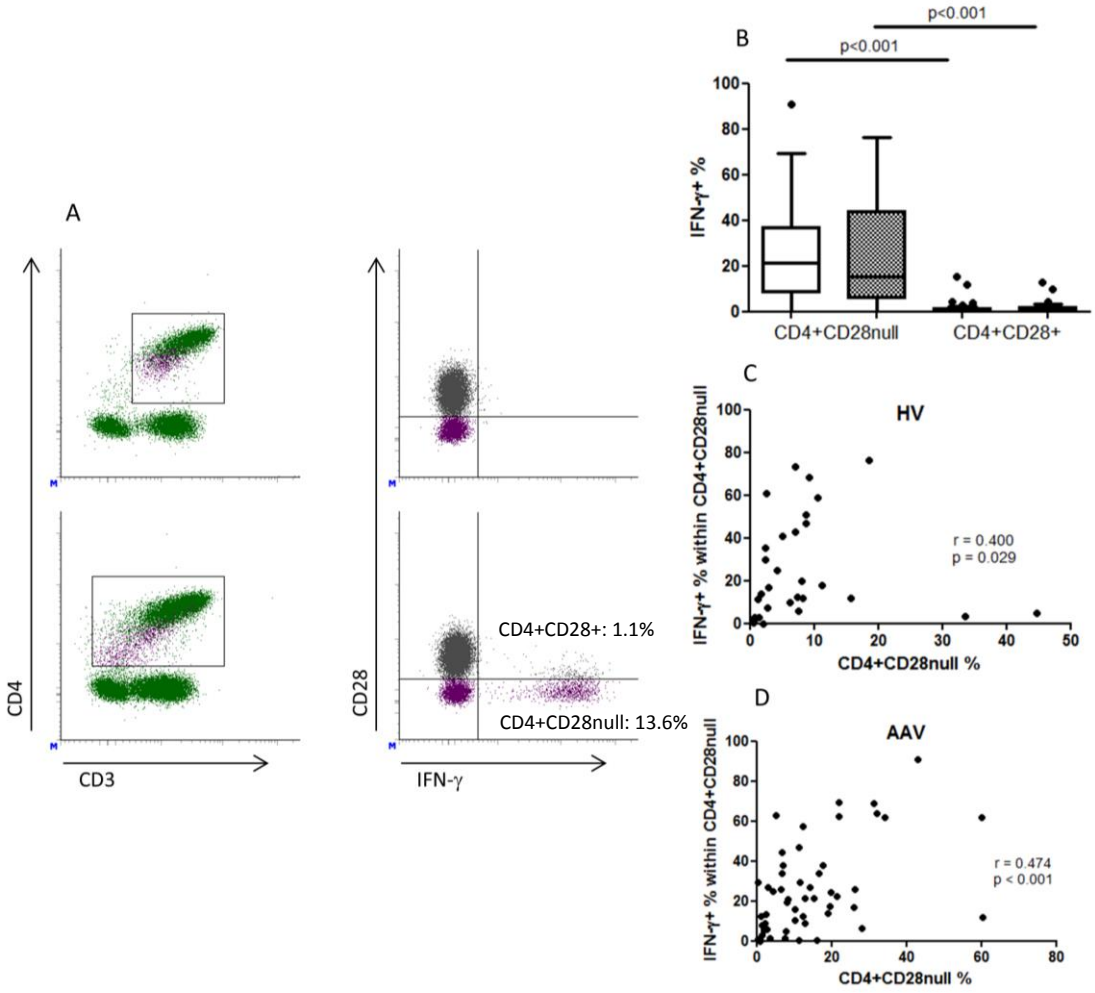

Figure 3.3 CD4+CD28null T-cell responsiveness to CMV lysate

Panel A: Gating strategy shown in a representative patient sample. PBMC were cultured overnight in the presence of CMV lysate (bottom row) or in culture medium alone (top row). CD4+CD28null T-cells are displayed in purple and CD4+CD28+ T-cells in grey colour. Panel B: A higher proportion of CD4+CD28null T-cells produced IFN-γ in response to CMV lysate compared to CD4+CD28+ T-cells (AAV (n=53) white boxes, HV (n=30) shaded boxes). Median and Tukey boxplots are shown; Wilcoxon matched pairs signed rank test employed for statistical comparisons. Panels C and D display the correlation between the size of the CD4+CD28null T-cell compartment and the proportion of CD4+CD28null T-cells that responded to CMV lysate by IFN-γ production after overnight culture (Spearman's rank test) in HV and AAV respectively.

3.4.1 The size of CD4+CD28null T-cell expansions is closely linked to the overall CMV response in the CD4 compartment

Given the fact that the origin of CD4+CD28null T-cells remains a subject of controversy in the literature [171, 184], measured variables including demographic parameters, renal function, the total CMV response in the CD4 compartment, CMV IgG titre and plasma levels of soluble markers of inflammation and endothelial dysfunction were related to the percentage of CD4+CD28null T-cells. On univariable analysis, age, the percentage of CD8CD28null T-cells, plasma IP-10 (the ligand for CXCR3) and the overall CMV response in the CD4 compartment (proportion of IFN-γ positive CD4 T-cells following overnight culture with CMV lysate) were significantly associated with the size of the CD4+CD28null T-cell expansion (Table 3.2).

Table 3.2 Univariable analysis of factors associated with the size of the CD4+CD28null T-cell expansion in AAV patients (n=53)

Variable	R value	p value
Age	0.346	0.011
Gender	0.164	0.241
eGFR	0.009	0.951
Creatinine	-0.069	0.625
uACR	-0.107	0.445
Total CMV response in CD4 compartment (Percentage of IFN-γ + cells)*	0.791	<0.001
CD4 count	-0.209	0.134
CD4 : CD8 ratio	-0.263	0.057
CD8CD28null %	0.525	<0.001
CMV IgG titre[a]	0.072	0.608
CRP[a]	-0.024	0.868
sICAM-1[a]	-0.162	0.251
sVCAM-1[a]	-0.157	0.265
Fractalkine[a]	0.132	0.353
IP-10[a]	0.380	0.005
RANTES[a]	0.061	0.665
E-Selectin[a]	0.125	0.379
P-Selectin[a]	0.226	0.107
IFN-γ[a]	0.044	0.759
TNF-α[a]	0.017	0.905
IL-2[a]	-0.041	0.771
MCP-1[a]	0.169	0.232
IL-6[a]	0.040	0.780
IL-12[a]	0.122	0.387
IL-17[a]	0.030	0.834
IL-10[a]	0.043	0.764

* Following overnight culture with CMV lysate; [a] Measured in plasma
uACR: urinary albumin creatinine ratio

A multivariable linear regression model was designed in order to determine which variables were independently associated with the size of the CD4+CD28null T-cell expansion in AAV patients after controlling for relevant covariates. All variables with a p value ≤ 0.1 on univariable analysis were entered in a multivariable linear regression model (Table 3.3). The size of the CD4 CMV specific compartment, plasma IP-10 and the proportion of CD8CD28null T-cells remained independently associated with the size of the CD4+CD28null T-cell expansion after controlling for possible confounders whilst age was not significant (Table 3.3). Graphical representation revealed a close linear relationship between CD4+CD28null T-cells proportion and the proportion of IFN-γ positive CD4 T-cells following CMV lysate stimulation (Figure 3.4) suggesting, together with the multivariable analysis results, that the size of the CD4+CD28null T-cell expansion is largely determined by the size of the overall CMV response in the CD4 compartment supporting the existing link in the literature between CMV infection and CD4+CD28null T-cell expansion.

Table 3.3 Multivariable analysis of factors associated with the size of the CD4+CD28null T-cell expansion in AAV patients

Variable	Univariable analysis	Multivariable analysis	
	p value	B value [95% CI]	p value
IFN-γ + % in CD4 compartment*	<0.001	1.03 [0.73, 1.32]	< 0.001
Age	0.011	0.18 [-0.05, 0.41]	0.120
CD4 : CD8 ratio	0.057	-0.98 [-2.68, 0.72]	0.253
CD8CD28null %	<0.001	0.15 [0.03, 0.28]	0.014
Plasma IP-10	0.005	0.01 [0.001, 0.01]	0.021

* Following overnight culture with CMV lysate
Multivariable model included all variables with a p value ≤ 0.1 on univariable analysis.
R value: 0.861
R Square: 0.742

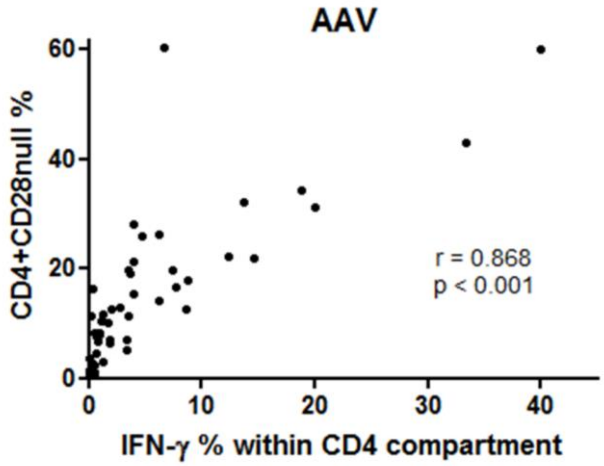

Figure 3.4 Correlation of CD4+CD28null T-cell proportion with the overall CD4 CMV response in AAV

The size of the overall CD4 CMV specific compartment identified by IFN-γ production following overnight culture in the presence of CMV lysate and monensin, closely correlated with the proportion of CD4+CD28null T-cells in the CD4 compartment (Spearman's rank test) (n=53).

3.4.2 Cytokine production by CD4+CD28null T-cells

The cytokine secreting potential of CD4+CD28null T-cells in response to CMV lysate was assessed following overnight culture in the presence of monensin followed by fixation / permeabilisation and intracellular staining for IFN-γ, TNF-α, IL-2, IL-5 and IL-10 as detailed in Section 2.6.5. An anti-CD154 monoclonal antibody conjugated to PE was also added in the overnight culture in order to identify activated T-cells. Staining from a representative patient sample is shown in Figure 3.5 including appropriate positive and negative controls.

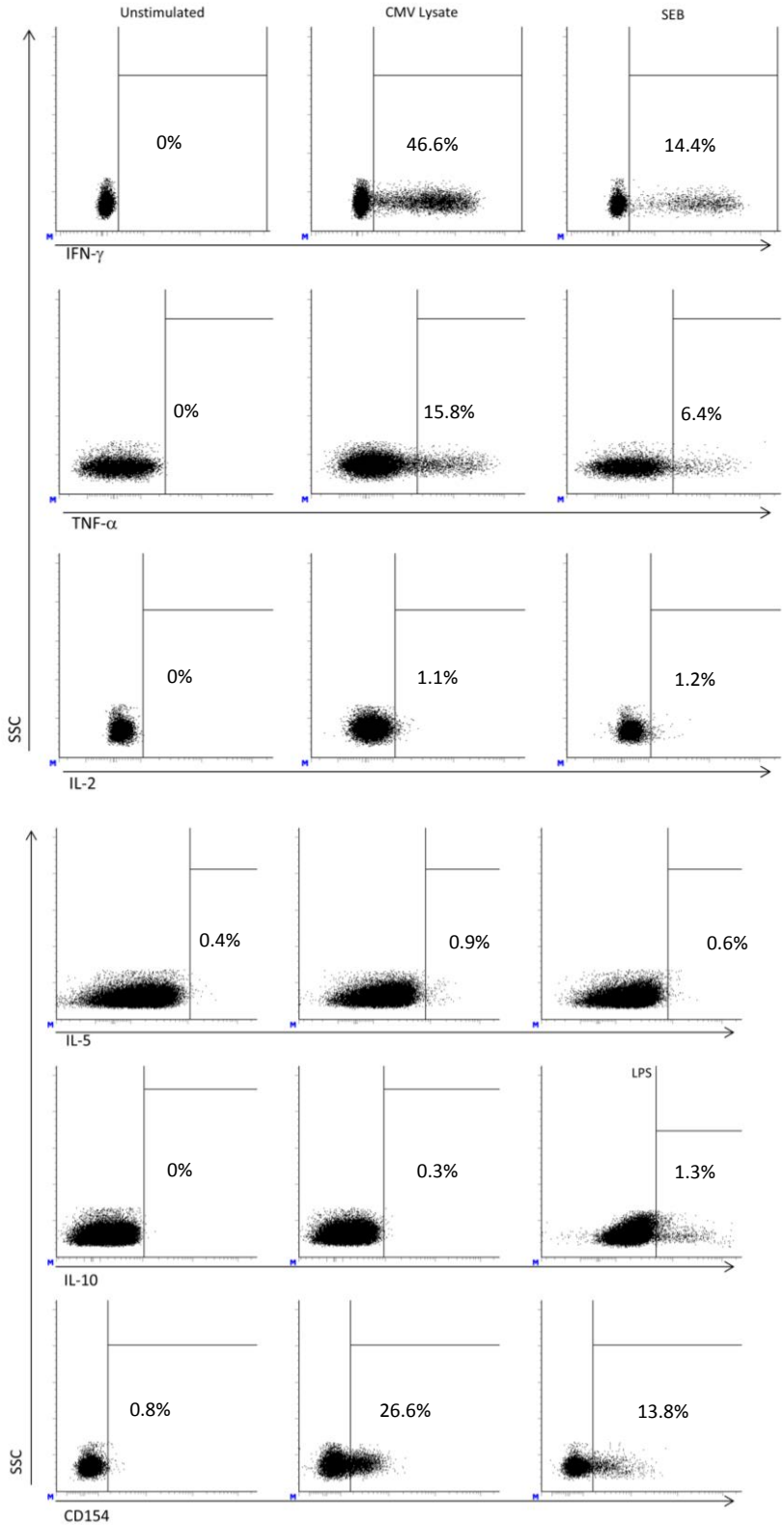

Figure 3.5 Cytokine expression in CD4+CD28null T-cells – representative staining

Staining from a representative patient sample for IFN-γ, TNF-α, IL-2, IL-5, IL-10 and the activation marker CD154 following overnight culture in medium alone (1st column), CMV lysate (2nd column) or SEB as a positive control (3rd column). Plots gated on CD4+CD28null T-cells. The IL-10 positive control was LPS instead of SEB as indicated and all CD4 T-cells are shown for that sample.

CD4+CD28null T-cells from AAV patients produced mainly IFN-γ and TNF-α following stimulation with CMV lysate (IFN-γ: 21.2 % [IQR 8.2 - 36.9]; TNF-α: 7.0 [2.5 - 12.9]). On the contrary, a much lower percentage was capable of IL-2 (0.4 [0.1 - 1.1]), IL-5 (0.6 [0.3 - 1.2]) and IL-10 (0.2 [0.1 - 0.5]) production. Interestingly, CD154 was detected on fewer cells than IFN-γ suggesting loss of CD154 during overnight culture. There was no difference between AAV and HC in the profile of the cytokines produced by CD4+CD28null T-cells (Figure 3.6).

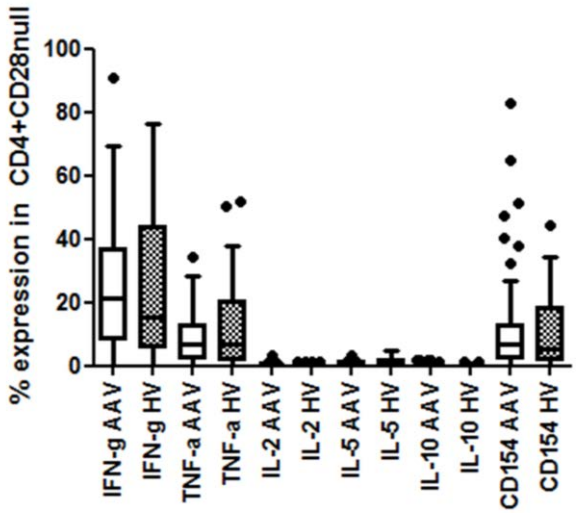

Figure 3.6 Cytokine expression in CD4+CD28null T-cells in AAV and HV

The proportion of CD4+CD28null T-cells expressing IFN-γ, TNF-α, IL-2, IL-5, IL-10 and the activation marker CD154 in AAV patients (n=53) (white boxes) and HV (n=30) (shaded boxes) after overnight co-culture with monensin, CD154 monoclonal antibody conjugated to PE and CMV lysate stimulation is shown.

3.4.3 Polyfunctionality of CD4+CD28null T-cells

Polyfunctionality of CD4+CD28null T-cells was assessed by quantifying the percentage of CMV responsive CD4+CD28null T-cells, identified by expression of IFN-γ, that were also capable of producing TNF-α and IL-2 following overnight stimulation with CMV lysate and comparing that to the functionality of CD4+CD28+ T-cells. The CD4+CD28null T-cell compartment contained significantly less polyfunctional cells within the IFN-γ positive CMV responsive compartment compared to CD4+CD28+ T-cells (AAV: CD4+CD28null 0.96 [IQR 0.16 - 2.31] versus CD4+CD28+ 5.19 [2.69 - 9.34]; $p<0.001$) (Figure 3.7). Therefore although a much greater proportion of CD4+CD28null T-cells were CMV responsive compared to their CD4+CD28+ counterparts, the CD4+CD28+ T-cell compartment contained a much higher proportion of polyfunctional CMV responsive T-cells as judged by their ability to secrete TNF-α and IL-2 in addition to IFN-γ suggesting that CD4+CD28+ CMV specific T-cells may confer a more robust immune response in the control of CMV infection. There was no difference in the proportions of polyfunctional CD4+CD28null or CD4+CD28+ T-cells between AAV patients and HV.

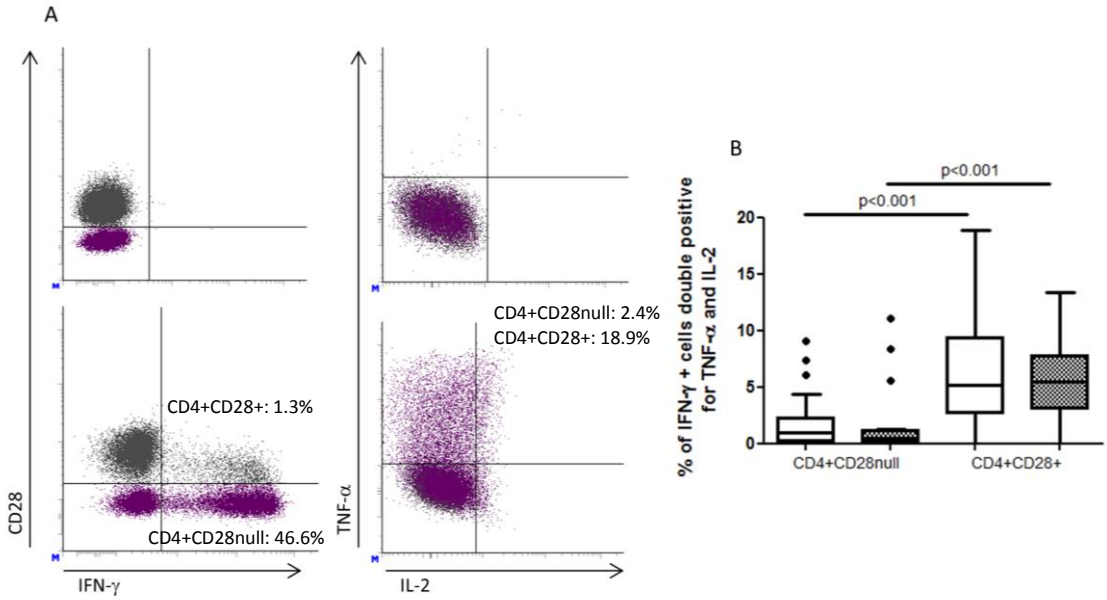

Figure 3.7 Polyfunctionality of CMV responsive CD4+CD28null T-cells

Polyfunctionality of CMV responsive CD4+CD28null T-cells was assessed by stimulating PBMC with CMV lysate in an overnight culture in the presence of monensin. CMV responsive CD4 T-cells were identified by IFN-γ expression. The proportion of T-cells also capable of expressing TNF-α and IL-2 within the CMV responsive compartment as shown in a representative patient sample in Panel A was determined for CD4+CD28null (purple) and CD4+CD28+ T-cells (grey). In Panel B, the proportions of polyfunctional CMV responsive T-cells within the CD4+CD28null and CD4+CD28+ compartments were compared (Wilcoxon matched pairs signed rank test) in AAV patients (n=53) (white boxes) and HV (n=30) (shaded boxes).

3.4.4 Inhibitory receptor expression by CD4+CD28null T-cells

Expression of the inhibitory receptors PD-1, TIM-3, LAG-3 and CTLA-4 has been associated with T-cell functional exhaustion and sequential loss of cytokine production [303, 337]. In order to further explore the functionality of CD4+CD28null T-cells, the expression of these inhibitory receptors was assessed following overnight culture of PBMC in the presence of CMV lysate. The proportion of CD4+CD28null T-cells expressing each of these receptors was compared to that of CD4+CD28+ T-cells in both AAV patients and HV. Isotype controls were used to guide gating (Figure 3.8).

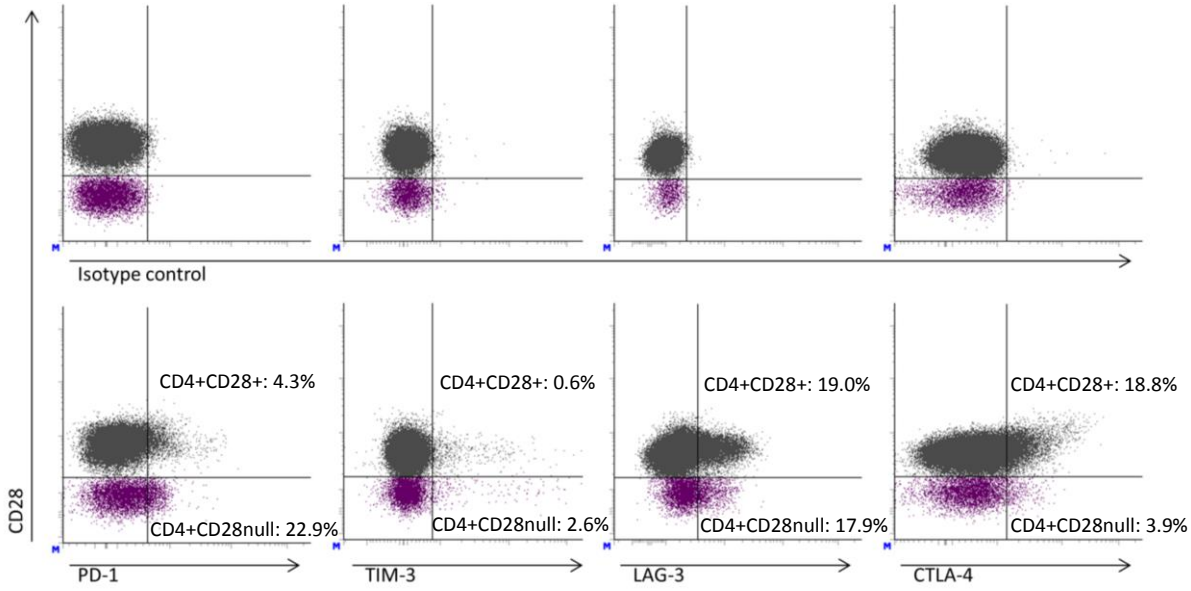

Figure 3.8 Inhibitory receptor expression in CD4+CD28null T-cells – representative plots

PBMC were stimulated overnight with CMV lysate and stained with surface and intracellular markers the next day in order to identify expression of the inhibitory receptors PD-1, TIM-3, LAG-3 and CTLA-4 on CD4+CD28null (purple) and CD4+CD28+ (grey) T-cells. Fluorescence minus one (FMO) experiments were also carried out where an isotype control was employed in place of the target antibody in order to assist with gating. The corresponding FMO experiment for each marker is shown in the top plot.

A significantly higher percentage of AAV CD4+CD28null T-cells compared to CD4+CD28+ T-cells expressed the inhibitory receptors PD-1 (9.5 [IQR 4.2 - 28.8] vs. 3.8 [2.8 - 7.1]; $p<0.001$) and TIM-3 (1.3 [0.9 - 2.5] vs. 0.6 [0.4 - 0.8]; $p<0.001$). There was no difference in the percentage of CD4+CD28null and CD4+CD28+ T-cells expressing

LAG-3 (14.4 [7.1 - 24.2] vs. 16.2 [9.2 - 22.2]; p=0.685) whereas a lower percentage of CD4+CD28null T-cells expressed CTLA-4 compared to CD4+CD28+ T-cells (9.4 [5.3 - 17.4] vs. 20.1 [15.5 - 25.7]; p<0.001). This pattern did not differ between AAV and HV. However, AAV patients overall tended to possess more CD4 T-cells expressing inhibitory receptors compared to HV (Figure 3.9).

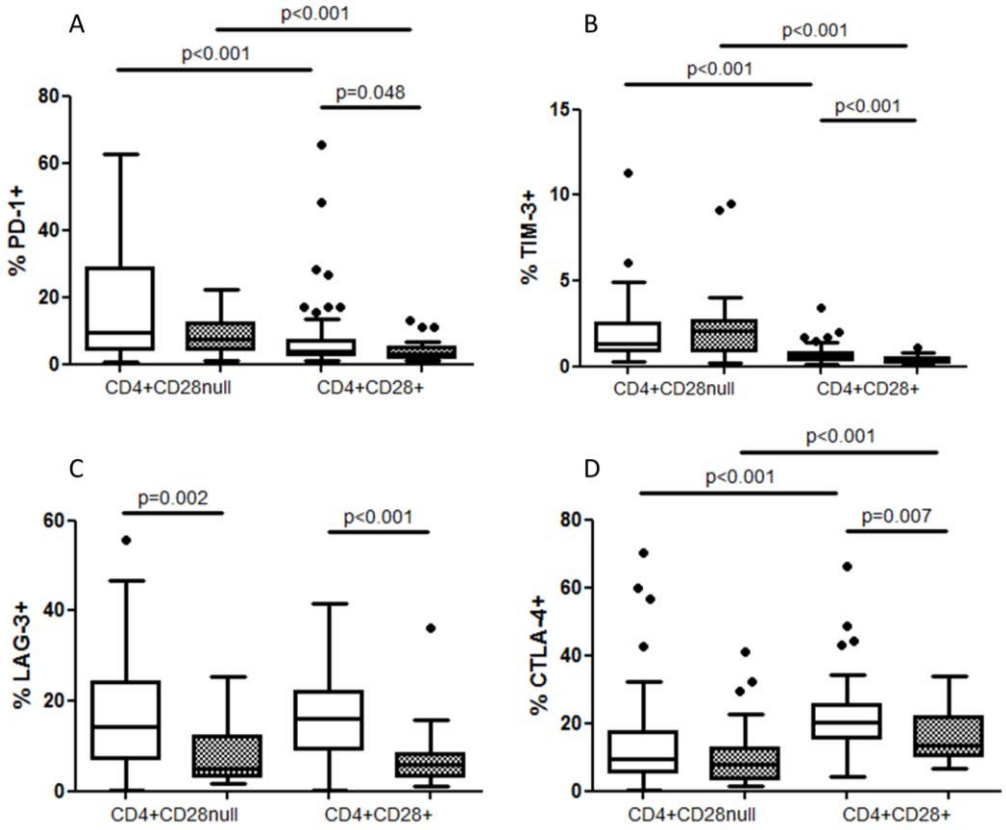

Figure 3.9 Inhibitory receptor expression in CD4+CD28null T-cells

The inhibitory receptor expression of PD-1 (Panel A), TIM-3 (Panel B), LAG-3 (Panel C) and CTLA-4 (Panel D) was compared between CD4+CD28null T-cells and CD4+CD28+ T-cells (Wilcoxon matched pairs signed rank test) and AAV (n=53) (white boxes) and HV (n=30) (shaded boxes) (Mann-Whitney U test).

Given the fact that CD4+CD28null T-cells displayed a lower degree of polyfunctionality and were more likely to express PD-1 and TIM-3 compared to their CD4+CD28+ counterparts, the expression of PD-1 and TIM-3 was compared between polyfunctional CD4+CD28null CMV specific T-cells and CD4+CD28null CMV specific T-cells capable of IFN-γ production only. Polyfunctional CMV specific CD4+CD28null T-cells were less likely to express TIM-3 compared to less functional CD4+CD28null T-cells whereas there was no difference in PD-1 expression (Figure 3.10), suggesting that expression of TIM-3 but not PD-1 on CD4+CD28null T-cells is associated with reduced polyfunctionality.

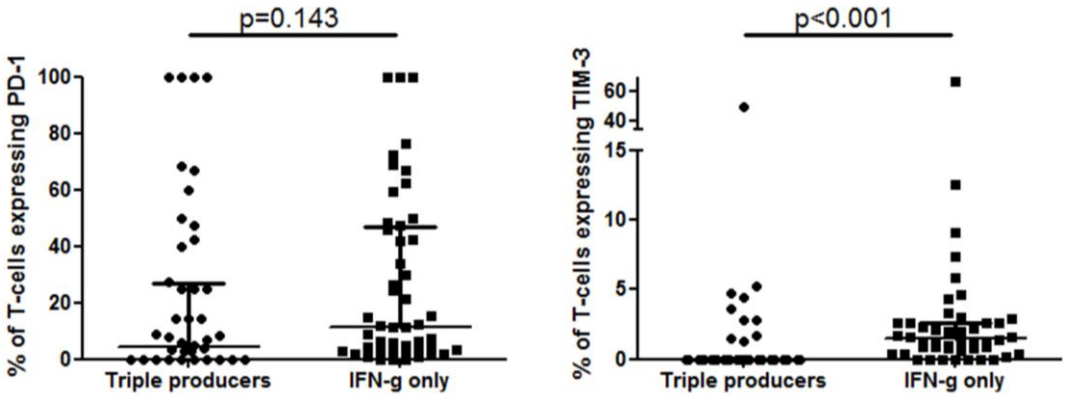

Figure 3.10 Expression of inhibitory receptors and polyfunctionality

The expression of the inhibitory receptors PD-1 and TIM-3 was compared between polyfunctional CD4+CD28null T-cells, identified as IFN-γ positive T-cells capable of TNF-α and IL-2 production, and CD4+CD28null T-cells capable only of IFN-γ production (Wilcoxon matched pairs signed rank test). Scatter dot plots with medians and IQR are shown.

3.4.5 T helper phenotype

The phenotype of CD4+CD28null T-cells was further explored by comparing the expression of the Th1 transcription factor T-bet in CD4+CD28null and CD4+CD28+ T-cells. In parallel, expression of the transcription factor BLIMP-1 was assessed (Figure 3.11). In chronic infections high expression of BLIMP-1 in CD8 T-cells has been shown to promote expression of inhibitory receptors and exhaustion whereas T-bet expression promotes functional effector responses [337].

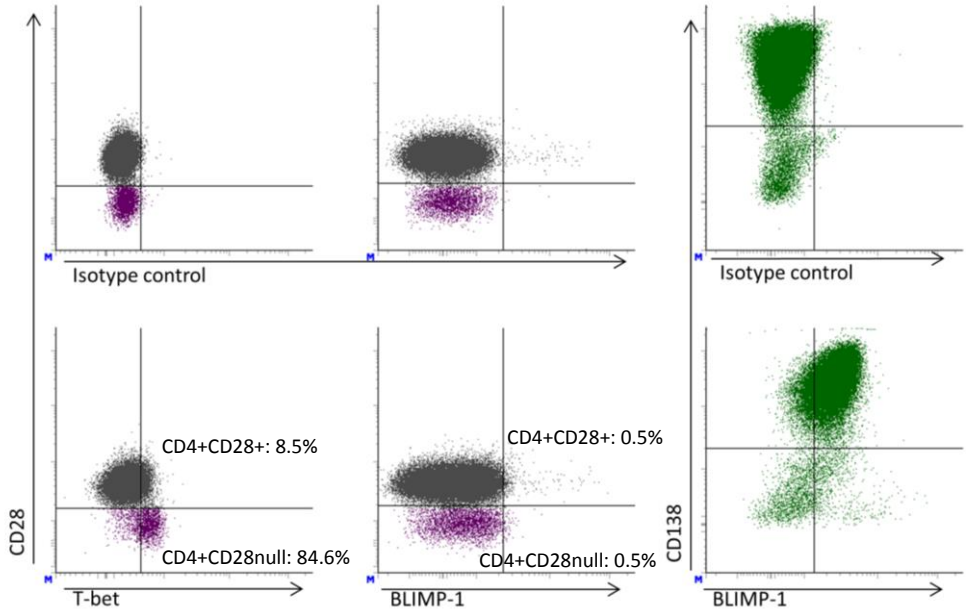

Figure 3.11 Expression of T-bet and BLIMP-1 – representative sample

Following overnight culture and stimulation with CMV lysate the expression of transcription factors T-bet and BLIMP-1 was assessed in CD4+CD28null (purple) and CD4+CD28+ (grey) T-cells as shown in this representative patient sample. FMO controls were employed to aid gating as shown in the top row. In addition, a myeloma cell line (U266), shown in the 3rd column, was stained with BLIMP-1 or isotype control as a positive control as myeloma cells constitutively express BLIMP-1. CD138 was used to identify plasma cells shown in green.

A large proportion of CD4+CD28null T-cells from both AAV patients and HV expressed T-bet compared to a relatively small percentage of CD4+CD28+ T-cells (Figure 3.12, Panel A). In AAV patients, 71.1 % of CD4+CD28null T-cells [IQR 53.4 - 81.4] expressed T-bet compared to 4.0 % of CD4+CD28+ T-cells [2.6 - 9.2] ($p<0.001$). Furthermore, the proportion of CD4+CD28null T-cells expressing T-bet was correlated to the size of the CD4+CD28null T-cell expansion ($r=0.773$, $p<0.001$) in an exponential relationship (Figure 3.12, Panel B) suggesting that expression of T-bet on CD4+CD28null T-cells increases as this subset expands. Conversely, BLIMP-1 expression was minimal in comparison to T-bet expression, although CD4 T-cells from AAV patients had a slightly higher expression of BLIMP-1 compared to HV (Figure 3.12, Panel A) mirroring the increased expression of inhibitory receptors seen in AAV patients in comparison to HV (Figure 3.9).

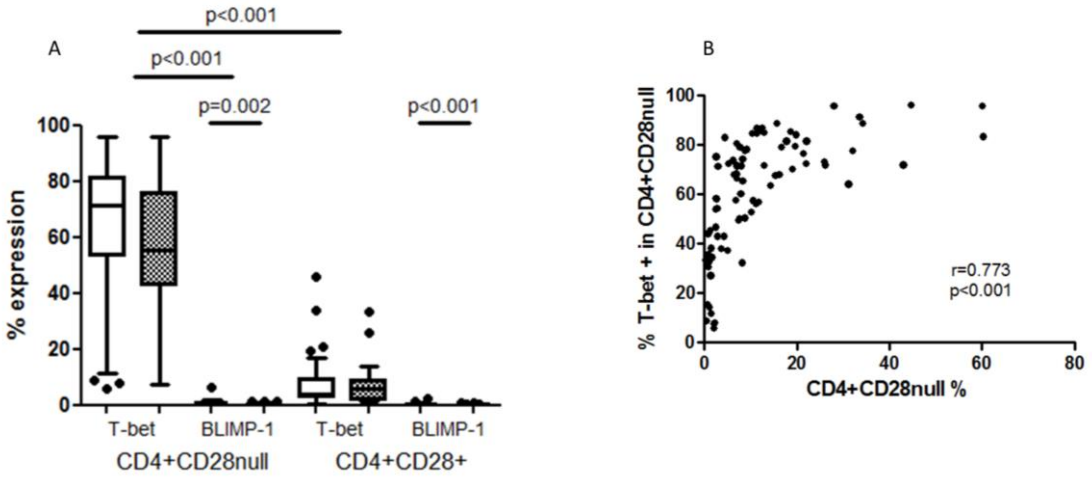

Figure 3.12 Expression of T-bet and BLIMP-1 – summary results

The proportion of CD4+CD28null and CD4+CD28+ T-cells expressing T-bet or BLIMP-1 was assessed in AAV patients (n=53) (white boxes) and HV (n=30) (grey boxes) (Panel A). A greater proportion of CD4+CD28null T-cells expressed T-bet compared to CD4+CD28+ T-cells in AAV and HV. CD4+CD28null T-cells were more likely to express T-bet than BLIMP-1 in both AAV and HV. CD4 T-cells from AAV patients were more likely to express BLIMP-1 compared to HV. Comparisons within AAV patients and within HV were performed using Wilcoxon matched pairs signed rank test whilst comparisons across AAV patients and HV were performed using the Mann-Whitney U test. In panel B, as the size of the CD4+CD28null T-cell expansion increased CD4+CD28null T-cells were more likely to express T-bet (n=83).

Given the fact that a high proportion of CD4+CD28null T-cells were found to express the Th1 transcription factor T-bet, the T helper subtype of CD4+CD28null T-cells was confirmed in a subset of AAV patients (n=17) and HV (n=6). Whole blood was stained for CXCR3, CCR4 and CCR6 (Figure 3.13) in order to identify Th1 (CXCR3+ CCR6-), Th17 (CCR4+ CCR6+), Th1Th17 (CXCR3+ CCR6+) and Th2 (CCR4+CCR6-) T-cells [338].

In keeping with T-bet expression, CD4+CD28null T-cells exhibited a Th1 type signature as identified by CXCR3 expression and minimal expression of CCR6 and CCR4. CD4+CD28null T-cells were significantly more likely to exhibit a Th1 pattern compared to CD4+CD28+ T-cells whereas the opposite was true for Th1Th17, Th17 and Th2 signatures (Figure 3.13). There was no difference in this pattern between AAV patients and HV. The high expression of CXCR3 by CD4+CD28null T-cells was in agreement with the plasma level of IP-10, the ligand for CXCR3, that was found to be independently associated with the size of the CD4+CD28null T-cell expansion on multivariable analysis (Table 3.3).

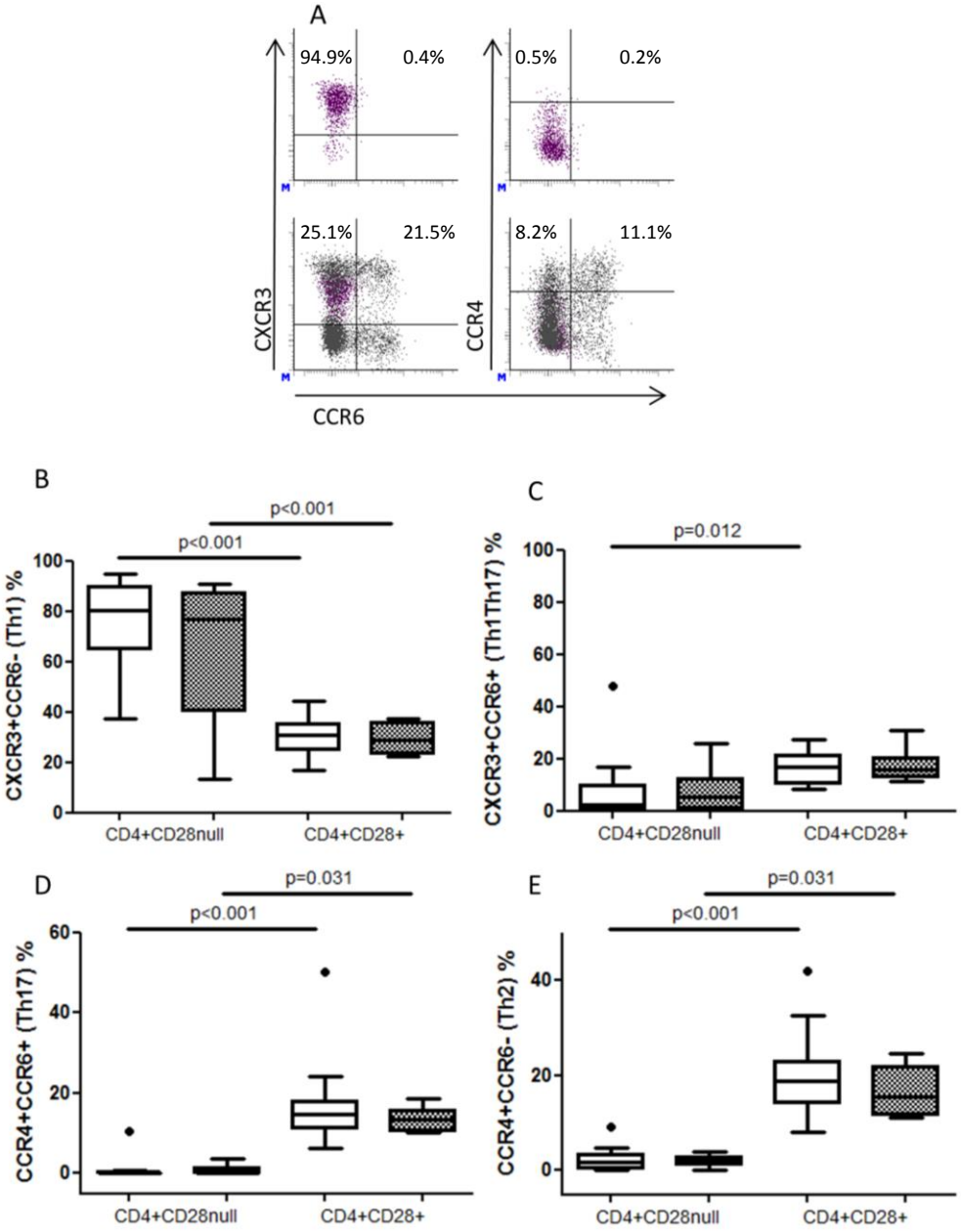

Figure 3.13 T helper phenotype of CD4+CD28null T-cells

Whole blood was stained with CXCR3, CCR4 and CCR6 antibodies in order to identify Th1, Th1Th17, Th17 and Th2 signatures in CD4+CD28null (purple) and CD4+CD28+ (grey) T-cells as seen in a representative patient sample in Panel A where the top row plots are gated on CD4+CD28null T-cells (purple) and the bottom row plots on CD4+CD28+ (grey) T-cells. Panel B shows summary results in AAV patients (n=17) (white boxes) and HV (n=6) (shaded boxes).

3.5 Discussion

CD4+CD28null T-cells in AAV patients were found to mainly produce IFN-γ and TNF-α in response to CMV lysate, in keeping with a Th1 subtype and in agreement with a previous report that described CD4+CD28null T-cells to be major sources of IFN-γ and TNF-α within granulomas in patients with GPA [35]. In contrast, these cells produced relatively small amounts of IL-2 and negligible IL-5 and IL-10. The Th1 phenotype of CD4+CD28null T-cells was further confirmed by staining for the chemokine receptor CXCR3 and the Th1 transcription factor T-bet that were present in the majority of CD4+CD28null T-cells. Furthermore CD4+CD28null T-cells did not express CCR4 or CCR6 seen in Th2 (CXCR3-CCR4+CCR6-) and Th17 cells (CXCR3-CCR4+CCR6+) respectively.

The proportion of CMV responsive T-cells amongst CD4+CD28null T-cells increased as the CD4+CD28null T-cell compartment expanded. Moreover, the magnitude of the CD4 CMV response displayed a very close linear correlation to the CD4+CD28null T-cell percentage and on multivariable analysis was the best independent determinant of the size of the CD4+CD28null T-cell expansion in AAV patients in disease remission.

Several studies have highlighted that the bimodal 'carrier' pattern of CD4+CD28null T-cells can be explained by past infection with CMV. In that respect Hooper *et al.* showed that in rheumatoid arthritis patients, CD4+CD28null T-cell expansions were

only present in CMV seropositive individuals [168]. Similarly, Morgan *et al.* described that significant expansions of CD4+CD28null T-cells were only present in CMV seropositive AAV patients [112].

CD4+CD28null T-cells expand with age and as CMV seroprevalence also increases with age it can often be difficult to disentangle the two from each other [184]. Furthermore, other researchers have suggested alternate antigens that may be responsible for the generation of CD4+CD28null T-cells such as heat shock proteins (HSP) [178, 200]. In these experiments, CD4+CD28null T-cells from patients with unstable angina, reacted to HSP60 but not CMV by upregulation of IFN-γ and perforin mRNA, casting doubt on the antigen specificity of these cells [200].

The data presented here lend strong support to the existing evidence in the literature that suggests CMV infection is the driving force behind CD4+CD28null T-cell expansion. Interestingly, age was not associated with the size of the CD4+CD28null T-cell compartment after controlling for relevant confounders suggesting that the total CMV CD4 response defines the CD4+CD28null T-cell expansion independently from age and is more important than age itself in that respect, at least in an immunosuppressed patient population with an inflammatory disease.

A limitation of this study in terms of defining the CMV specificity of CD4+CD28null T-cells is that only CMV seropositive patients were included. This however was deliberate as significant CD4+CD28null T-cell expansions greater than 2% of the CD4 compartment are only present in the setting of CMV seropositivity [112] and the primary objective of this chapter was to phenotype this cell type in AAV rather than prove that they are CMV specific. For this reason CD4+CD28null cells were also not challenged with alternative antigens during the course of this work.

Previous experiments have shown that CD4+CD28null T-cells proliferate in response to CMV antigens but not in response to other antigens like varicella zoster, tetanus toxoid and tuberculin purified protein derivative [112, 184], although as mentioned earlier reactivity to HSP60 has been demonstrated [200]. Bason and colleagues have shown that a short sequence of HSP60 shares homology with two CMV encoded proteins and that CMV infection induces antibodies that are able to bind HSP60 and cause endothelial damage [236]. It would be interesting to determine whether CMV specific CD4+CD28null T-cells are also able to recognise both CMV antigens and HSP60 and whether dual recognition is associated with increased cytotoxicity.

The phenotype of CD4+CD28null T-cells was similar in AAV patients in remission and age matched healthy volunteers. AAV patients had a significantly higher proportion of CD4+CD28null T-cells within their CD4 compartment although there was no difference in the absolute count as AAV patients were lymphopoenic, as expected. AAV patients

are heavily immunosuppressed and therefore it is possible that subclinical CMV reactivation is more frequent in these patients compared to healthy people thereby leading to an exaggerated proportionate expansion of CD4+CD28null T-cells.

In order to determine whether CD4+CD28null T-cells possess aspects of functionally exhausted cells, their capacity to produce multiple cytokines was assessed. A lower proportion of CD4+CD28null T-cell CMV specific cells were polyfunctional as judged by production of all three IFN-γ, TNF-α and IL-2 cytokines compared to their CD4+CD28+ CMV specific counterparts. In a bone marrow transplantation study patients that possessed polyfunctional CD8 T-cells were protected from CMV reactivation [339]. CD4 T-cells capable of producing multiple cytokines are thought to be more effective than single-cytokine producing cells [304] in part due to their ability to sustain CD8 responses by IL-2 secretion [340]. Although much less is known about the importance of polyfunctional CD4 T-cells in the control of CMV reactivation, recently it has been shown that following lung transplantation CMV specific CD4 T-cells able to co-produce IFN-γ, TNF-α and IL-2 conferred protection from subsequent CMV viraemia whereas single producers of IFN-γ were detrimental [341]. Taken together with the current findings this suggests that the balance between CD4+CD28+ and CD4+CD28null CMV specific T-cells may be important in the control of CMV reactivation. There is currently no information in the literature on the effect of polyfunctional responses on subclinical CMV reactivation and hence this was further explored in the studies described in Chapter 5.

In an effort to explore potential functional exhaustion in CD4+CD28null T-cells further, their expression of inhibitory receptors and the transcription factor BLIMP-1 that are implicated in functional exhaustion was evaluated and compared to their CD4+CD28+ counterparts. A greater proportion of CD4+CD28null T-cells expressed PD-1 and TIM-3 compared to CD4+CD28+ cells although the expression of CTLA-4 was noted to be higher on CD4+CD28+ T-cells. There was no difference in the expression of LAG-3 or BLIMP-1 between CD28null and CD28+ cells and it is noteworthy that the expression of BLIMP-1 in CD4+CD28null T-cells was negligible. The transcriptional pathways that regulate functional exhaustion in CD4 T-cells are incompletely understood and it is likely that substantial heterogeneity exists amongst potentially exhausted CD4 T-cells [342]. Given that CD4+CD28null T-cells were more likely to express PD-1 and TIM-3 and exhibited reduced polyfunctionality compared to CD4+CD28+ cells, the proportion of polyfunctional CMV specific CD4+CD28null that expressed PD-1 or TIM-3 was compared to that of IFN-γ only producing CD4+CD28null T-cells. Single producers of IFN-γ were more likely to express the inhibitory receptor TIM-3 compared to polyfunctional CD4+CD28null T-cells whereas no difference was noted in PD-1 expression. This indicates that the expression of TIM-3 but not PD-1 on CD4+CD28null T-cells is associated with reduced functionality and identifies a subset of potentially exhausted less functional T-cells within the CD4+CD28null T-cell compartment.

TIM-3 has been linked to functional exhaustion and reduced functionality of T-cells in cancer [343] and hepatitis B infection [344] but has not been previously identified as a

marker of reduced function on CMV specific T-cells. This is of potential clinical importance both in the context of CMV reactivation and as expansions of CD4+CD28null T-cells have been previously linked to increased infections in patients with AAV [112] and reduced responsiveness to the influenza vaccine [300, 317]. However, it should be noted that the proportion of TIM-3 expressing CD4+CD28null T-cells was very low casting uncertainty onto the biological significance of this molecule in the control of functional anti-CMV responses. Indeed, expression of TIM-3 as well as other inhibitory receptors such as PD-1 may alternatively reflect recent lymphocytic activation by way of recent encounter with CMV antigens.

In summary, the work presented in this chapter has confirmed that CD4+CD28null T-cells in AAV represent a CMV responsive, Th1 proinflammatory subset. The data here has strengthened the association between CMV infection and CD4+CD28null T-cells by showing that the size of the CMV CD4 response determines the magnitude of the CD4+CD28null T-cell expansion in AAV patients and has indicated that CMV specific CD4+CD28+ T-cells are more polyfunctional compared to CMV specific CD4+CD28null T-cells. Finally, for the first time, TIM-3 expression on the surface of CMV specific CD4+CD28null T-cells has been identified as a marker of a functionally exhausted subset of CD4+CD28null T-cells.

CHAPTER 4

ASSOCIATION BETWEEN CD4+CD28null T-CELL EXPANSIONS AND CARDIOVASCULAR RISK IN ANCA ASSOCIATED VASCULITIS

Chapter 4 Association between CD4+CD28null T-cell expansions and cardiovascular disease risk in AAV

4.1 Introduction

CD4+CD28null T-cells have been persistently implicated in cardiovascular disease (CVD). They have been found to preferentially infiltrate unstable plaques [227], are present in higher proportions in unstable compared to stable angina patients and are thought to be involved in the pathophysiology of endothelial damage due to their potent production of IFN-γ [226]. *In vitro*, they have been shown to exhibit perforin dependent cytotoxic activity against human umbilical vein endothelial cells [228]. In addition, CD4+CD28null T-cells have been shown to be associated with endothelial dysfunction, identified by flow mediated vasodilatation, in rheumatoid arthritis (RA) patients [175] and have also been implicated in early atherosclerotic damage, identified by increased carotid artery intima-media thickness, in RA [175], chronic kidney disease (CKD) [179, 180] and haemodialysis patients [181].

Cardiovascular disease is a leading cause of mortality in AAV [93] and CD4+CD28null T-cells have previously been linked with increased mortality in this patient group [112]. In addition, CMV seropositivity has been associated with increased arterial stiffness, an established marker of CVD risk, in patients with CKD [285].

The data presented in Chapter 3 confirmed that expansions of CD4+CD28null T-cells in AAV patients are CMV responsive and exhibit a Th1, pro-inflammatory phenotype. The work presented in this chapter aimed to:

- Determine the impact of CD4+CD28null T-cells on CVD risk in AAV by assessing their relationship to arterial stiffness and blood pressure parameters and
- Explore the mechanisms via which CD4+CD28null T-cells may contribute to CVD in inflammatory disease by assessing their expression of endothelial receptors and markers of cytotoxicity in patients with AAV

Blood pressure and arterial stiffness parameters were measured on 53 CMV seropositive patients with stable AAV in remission. CD4+CD28null T-cells were enumerated on the same day by whole blood staining as detailed in Chapter 2. The AAV cohort characteristics have been discussed in Chapter 3 (Table 3.1).

4.2 CD4+CD28null T-cell expansions correlate with increased systolic blood pressure and pulse pressure

The size of the CD4+CD28null T- cell expansion was significantly correlated to systolic blood pressure measured peripherally and centrally (Figure 4.1, Panels A and C). No correlation was seen between CD4+CD28null T-cell percentage and diastolic blood pressure or mean arterial pressure (MAP) (Figure 4.1, Panels B and D).

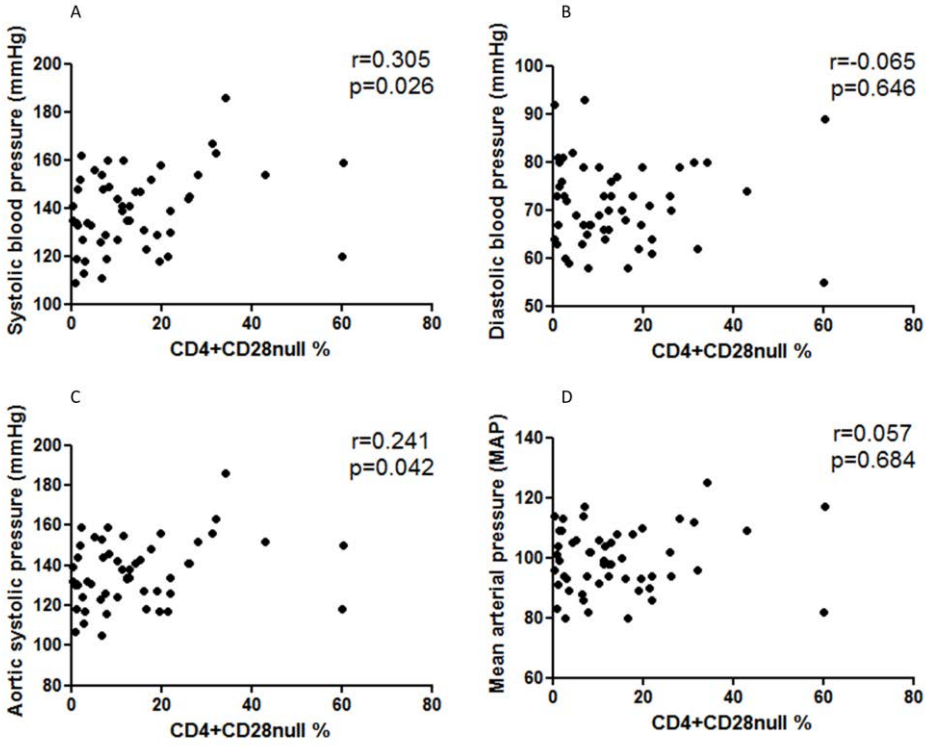

Figure 4.1 CD4+CD28null T-cell expansions and blood pressure parameters. Correlations (Spearman rank test) are shown between the size of the CD4+CD28null T-cell expansion in CMV seropositive AAV patients in remission (n=53) and peripheral systolic blood pressure (A), peripheral diastolic blood pressure (B), aortic systolic pressure (C) and mean arterial pressure (D).

Pulse pressure, the difference between systolic and diastolic blood pressure, is a surrogate marker of arterial stiffness and is increased in atherosclerosis and arteriosclerosis. It is has been shown to be a better predictor of cardiovascular outcomes compared to mean arterial pressure and diastolic blood pressure [345, 346]. The percentage of CD4+CD28null T-cells in AAV was significantly correlated with both peripheral and central pulse pressure in that patients with larger expansions of CD4+CD28null T-cells exhibited a higher pulse pressure (Figure 4.2).

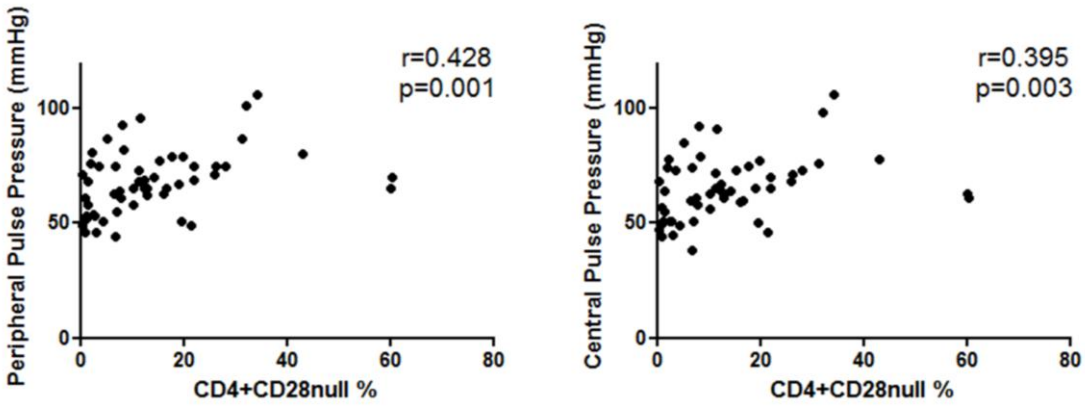

Figure 4.2 CD4+CD28null T-cells and pulse pressure

Correlations (Spearman rank test) are shown for CD4+CD28null T-cells from CMV seropositive AAV patients in remission (n=53) and peripheral (left) and central (right) pulse pressure.

4.3 The CD4+CD28null T-cell percentage is independently associated with increased arterial stiffness

Next, the percentage of CD4+CD28null T-cells in AAV patients was related to the gold standard measure of arterial stiffness, pulse wave velocity (PWV). Aortic PWV is a powerful independent predictor of all-cause mortality and cardiovascular events in ESRD, hypertensive subjects, diabetic patients and the general population [347, 348]. Carotid to femoral aortic PWV was measured as described in Chapter 2. In keeping with the observed relationship between CD4+CD28null T-cells and pulse pressure, the size of the CD4+CD28null T-cell expansion was found to be correlated with increased PWV (Figure 4.3).

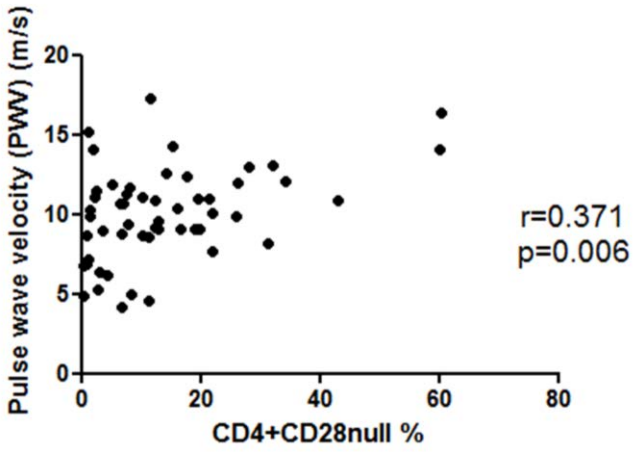

Figure 4.2 CD4+CD28null T-cells and pulse wave velocity

Correlation (Spearman rank test) is shown for CD4+CD28null T-cells from CMV seropositive AAV patients in remission (n=53) and pulse wave velocity.

Univariable analysis revealed that PWV was associated with several other factors that could potentially confound the observed association with CD4+CD28null T-cell percentage (Table 4.1).

Table 4.1 Factors associated with PWV on univariable analysis (n=53)

Variable	Univariable analysis		
	R Square	B value [95% CI]	p value
Age (years)	0.183	0.131 [0.053, 0.208]	0.001
Gender	0.004	0.393 [-1.313, 2.100]	0.645
Creatinine μmol/L	0.021	0.008 [-0.008, 0.025]	0.305
eGFR (mL/min/1.73m^2)	0.026	-0.023 [-0.062, 0.017]	0.252
Urinary Albumin Creatinine Ratio (ACR)	0.056	0.019 [-0.003, 0.041]	0.088
Peripheral Pulse Pressure (mmHg)	0.163	0.084 [0.030, 0.137]	0.003
Central Pulse Pressure (mmHg)	0.126	0.077 [0.019, 0.135]	0.010
Mean Arterial Pressure (mmHg)	0.102	0.088 [0.015, 0.161]	0.020
Systolic Blood Pressure (mmHg)	0.191	0.079 [0.033, 0.124]	0.001
Diastolic Blood Pressure (mmHg)	0.027	0.056 [-0.039, 0.150]	0.243
Aortic Systolic Pressure (mmHg)	0.162	0.074 [0.026, 0.122]	0.003
CD4+CD28null T-cell proportion (%)	0.182	0.091 [0.037, 0.146]	0.001
C-reactive protein	0.008	1.169×10^{-7} [0.000, 0.000]	0.520
RANTES	0.004	-2.048×10^{-5} [0.000, 0.000]	0.676
sICAM-1	0.005	5.27×10^{-7} [0.000, 0.000]	0.625
sVCAM-1	0.004	1.18×10^{-6} [0.000, 0.000]	0.638
IL-2	0.066	0.007 [0.000, 0.015]	0.065
MCP-1	0.016	-0.002 [-0.007, 0.003]	0.366
Fractalkine	0.009	0.020 [-0.039, 0.078]	0.5060
E-selectin	0.001	3.34×10^{-6} [0.000, 0.000]	0.866
IFN-γ	0.036	-0.004 [-0.010, 0.002]	0.179
IL-10	0.025	0.089 [-0.068, 0.245]	0.260
IL-6	0.004	0.002 [-0.007, 0.011]	0.649
TNF-α	0.083	0.015 [0.001, 0.029]	0.039
IP-10	0.006	-0.001 [-0.002, 0.001]	0.586
P-selectin	0.015	9.66×10^{-6} [0.000, 0.000]	0.395
IL-17	0.015	-0.003 [-0.011, 0.004]	0.389
IL-12	0.001	0.008 [-0.059, 0.074]	0.818

In order to determine whether the size of the CD4+CD28null T-cell expansion was independently associated with increased PWV, variables linked to PWV with a p value less than or equal to 0.1 on univariable analysis were entered in a multivariable linear regression model (Table 4.2). The CD4+CD28null T-cell percentage was independently associated with PWV after controlling for relevant confounders. The only other factor that remained significantly associated with PWV was age.

In order to avoid collinearity, only one blood pressure parameter was added at each iteration of the model. Table 4.2 shows the final model with MAP. The CD4+CD28null T-cell percentage remained independently associated with PWV with minimal changes in the overall R square, B values and p values when peripheral pulse pressure, central pulse pressure, peripheral systolic blood pressure or central aortic blood pressure were substituted for MAP. Similarly, the final model shown in Table 4.2 includes TNF-α but not IL-2 in order to avoid significant collinearity. There was no change when TNF-α was substituted for IL-2 and the size of the CD4+CD28null T-cell expansion remained independently associated with PWV.

Table 4.2 Multivariable model analysis of CD4+CD28null T-cell expansion and PWV

Variable	Univariable analysis	Multivariable analysis	
	p value	**B value [95% CI]**	**p value**
CD4+CD28null T-cell proportion	0.001	0.066 [0.013, 0.119]	0.016
Age	0.001	0.080 [0.006, 0.155]	0.035
Urinary ACR	0.088	0.013 [-0.007, 0.033]	0.196
Mean arterial pressure (MAP)	0.020	0.053 [-0.016, 0.122]	0.128
TNF-α	0.039	0.010 [-0.002, 0.022]	0.086

Multivariable model included all variables with a p value ≤ 0.1 on univariable analysis.

R value: 0.635
R Square: 0.404

4.4 CD4+CD28null T-cell expression of endothelial receptors and markers of cytotoxicity

Given the observed association between CD4+CD28null T-cell percentage and increased arterial stiffness, the expression of the endothelial receptors CX3CR1 (receptor for fractalkine), CD49d (receptor for VCAM-1) and CD11b (receptor for ICAM-1) was assessed on CD4+CD28null T-cells and compared to the expression of these receptors on CD4+CD28+ T-cells. In parallel, the expression of the intracellular

cytotoxic molecules granzyme B and perforin was assessed as a marker of cytotoxic potential. These experiments were performed on cryopreserved PBMC in a random subset of AAV patients (n=10).

A large percentage of CD4+CD28null T-cells expressed all three endothelial receptor molecules compared to CD4+CD28+ T-cells (51.6 [IQR 42.7 - 64.4] vs. 5.0 [1.7 - 6.3]; p=0.006). Similarly, the majority of CD4+CD28null T-cells were cytotoxic, as defined by the expression of both granzyme B and perforin, compared to a very low proportion of CD4+CD28+ T-cells (74.5 [63.5 - 92.7] vs. 2.9 [1.8 - 5.4]; p=0.016; Wilcoxon matched pairs signed rank test) (Figure 4.3).

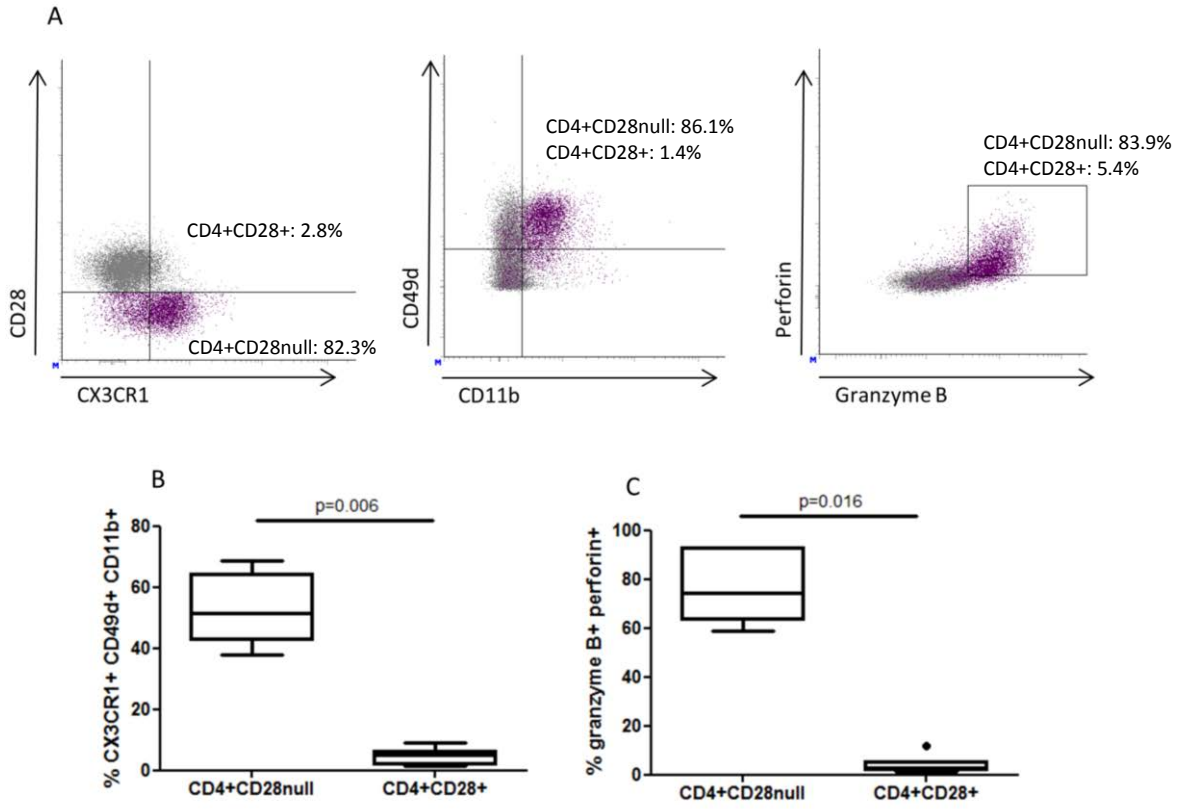

Figure 4.3 Expression of endothelial receptors and markers of cytotoxicity

Cryopreserved PBMC from 10 AAV patients were thawed and stained for expression of the endothelial receptors CX3CR1, CD49d and CD11b as well as the intracellular cytotoxic molecules granzyme B and perforin. Panel A shows staining from a representative patient sample where CD4+CD28null T-cells are shown in purple and CD4+CD28+ T-cells in grey. In Panel B the proportion of CD4+CD28null T-cells expressing all 3 endothelial receptors and in Panel C the proportion expressing both granzyme B and perforin were compared to those of CD4+CD28+ T-cells.

Next, the expression of the activating natural killer cell receptor NKG2D on CD4+CD28null T-cells and its co-expression with CX3CR1, CD49d, CD11b and the cytotoxic molecules granzyme B and perforin was assessed as NKG2D has previously been implicated in endothelial cytotoxicity [225]. This again was performed in a random subset of AAV patients (n=10).

A much higher percentage of CD4+CD28null T-cells expressed NKG2D compared to their CD4+CD28+ counterparts (27.4 [IQR 12.8 - 44.9] vs. 2.8 [0.8 - 5.1]; p=0.002) (Figure 4.4). In addition, the majority of the NKG2D positive CD4+CD28null T-cell compartment expressed CX3CR1 (82.7% [75.0 - 90.4]) and pre-formed cytotoxic molecules granzyme B and perforin (85.7 [68.8 - 97.2]). A large percentage of NKG2D+ CD4+CD28null T-cells were dual positive for CD49d and CD11b expression (38.2 [20.8 - 51.9]).

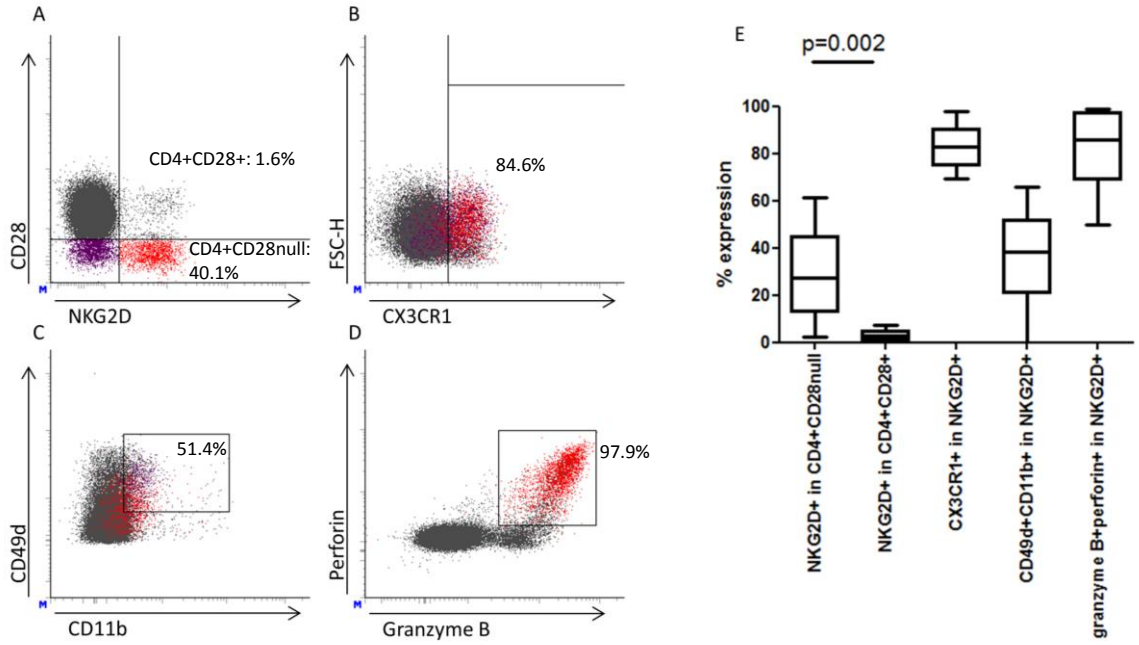

Figure 4.4 NKG2D staining in CD4+CD28null T-cells

Cryopreserved PBMC from 10 AAV patients were thawed and stained with surface and intracellular markers to identify NKG2D positive cells within the CD4+CD28null T-cell compartment and investigate their phenotype in terms of CX3CR1 expression (Panel B), CD49d CD11b double expression (Panel C) and granzyme B perforin double expression (Panel D). CD4+CD28null T-cells are depicted in purple, CD4+CD28+ cells in grey and NKG2D+ CD4+CD28null cells in red (Panels A-D). Panel E shows summary results comparing the proportion of CD4+CD28null T-cells and CD4+CD28+ T-cells expressing NKG2D and proportions of NKG2D+ CD4+CD28null T-cells co-expressing CX3CR1, CD49d and CD11b as well as granzyme B and perforin.

4.5 Discussion

Having shown that CD4+CD28null T-cells in AAV are CMV responsive, pro-inflammatory T-cells, work presented in this chapter aimed to characterise these cells further in terms of their cytotoxic potential and expression of endothelial homing receptors and investigate their relevance in contributing to cardiovascular risk in patients with AAV where cardiovascular disease (CVD) is a major source of morbidity and mortality.

The size of the CD4+CD28null T-cell expansion in AAV patients in remission was shown to be correlated with multiple measures of blood pressure and arterial stiffness. In addition, after controlling for relevant confounding variables such as age, mean arterial pressure, proteinuria and levels of systemic inflammation, the CD4+CD28null T-cell percentage was independently associated with carotid to femoral PWV, the gold standard measure of arterial stiffness and a well-established surrogate marker of cardiovascular outcomes.

The data presented in Chapter 3 showed that CD4+CD28null T-cells in AAV patients are Th1 proinflammatory cells by virtue of their abundant secretion of IFN-γ and TNF-α and expression of T-bet and CXCR3. These observations are extended here to show that the majority of CD4+CD28null T-cells possess the endothelial homing receptors CX3CR1, CD49d and CD11b on their surface. Furthermore, the majority of

CD4+CD28null T-cells were found to contain the pre-formed cytotoxic molecules granzyme B and perforin.

The chemokine receptor CXCR3 is known to be responsible for the specific recruitment of Th1 cells to inflammatory sites [349]. In addition, its ligand, IP-10 and the CX3CR1 ligand, fractalkine, are expressed by activated endothelial cells and *in vitro* experiments have shown that following stimulation with CMV antigens, CMV effector cells are able to produce soluble factors that induce endothelial activation and production of IP-10 and fractalkine, migrate in an IP-10 / fractalkine dependent fashion and induce endothelial cell lysis [287]. The endothelium is a target tissue for CMV infection [350, 351] and therefore endothelial receptors on CMV specific cells may serve to target them to areas of potential reactivation.

Taken together, the data presented here suggests that expression of CXCR3, CX3CR1, CD49d and CD11b enables CD4+CD28null T-cells to target areas of inflamed endothelium through their respective ligands IP-10, fractalkine, VCAM-1 and ICAM-1 leading to perforin and granzyme B mediated endothelial cell lysis and vascular damage. Interestingly, the plasma concentration of IP-10 was found to be independently associated with the size of the CD4+CD28null T-cell expansion during the studies presented in Chapter 3 in keeping with possible endothelial upregulation of IP-10 in the setting of large CD4+CD28null T-cell expansions.

In addition, CD4+CD28null T-cells in AAV patients were found to express NKG2D an activating receptor usually found in natural killer cells as well as activated CD8 and CD4 T-cells and implicated in the targeting and lysis of glomerular endothelial cells [225]. NKG2D positive CD4+CD28null T-cells in turn were almost all CX3CR1 positive and almost always contained pre-formed granzyme B and perforin cytotoxic molecules.

Data presented in the previous chapter showed that in AAV patients, both the proportion of T-bet expressing and IFN-γ producing CD4+CD28null T-cells increase as the size of the CD4+CD28null T-cell expansion increases. This is also likely to be relevant in the mediation of vascular damage by this T-cell subset as T-bet deficient mice display a lack of infiltration at pathologic sites and are protected from a wide range of inflammatory diseases [352, 353] whilst both T-bet and IFN-γ are essential in the generation of angiotensin II mediated vascular dysfunction [354].

CD4+CD28null T-cell expansions have been linked with measures of early atherosclerotic damage such as increased carotid artery intima media thickness and endothelial dysfunction in patients with CKD and RA [175, 180, 181]. To date there has only been one study evaluating the contribution of CD4+CD28null T-cells to arterial stiffness in HIV infected women where the proportion of CD4+CD28null T-cells was shown to correlate with decreased carotid artery distensibility as assessed by B-mode ultrasound [355]. CMV seropositivity on the other hand has previously been

associated with increased arterial stiffness in CKD patients [285] but the way in which this effect is mediated is not clear.

The data presented in this thesis so far show that in CMV seropositive patients with an inflammatory disease CD4+CD28null T-cell expansions are comprised of CMV responsive, proinflammatory T-cells that possess multiple endothelial homing receptors and markers of cytotoxicity and have the capacity to potentially target and lyse endothelial cells by the production of granzyme B and perforin. Furthermore in the same cohort of patients, large expansions of these cells are independently linked to increased arterial stiffness likely accounting for the increased mortality seen in patients with AAV that possess increased expansions of CD4+CD28null T-cells. This implicates CMV in the exacerbation of vascular damage in inflammatory disease mediated by CD4+CD28null T-cells and opens new therapeutic opportunities not only in AAV but also in other inflammatory conditions such as CKD and RA where similar associations with CD4+CD28null T-cells and CVD have been observed.

CHAPTER 5

THE POTENTIAL FOR VALACICLOVIR TO PREVENT CMV MEDIATED ADVERSE MODULATION OF THE IMMUNE SYSTEM IN ANCA ASSOCIATED VASCULITIS (CANVAS)

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A RANDOMISED CONTROLLED OPEN LABEL PROOF OF CONCEPT CLINICAL TRIAL

Chapter 5 The potential for valaciclovir to prevent CMV mediated adverse modulation of the immune system in ANCA Associated VASculitis (CANVAS): a randomised controlled open label proof of concept clinical trial

5.1 Introduction

Multiple lines of evidence suggest that chronic CMV infection, and its association with an expanded population of CD4+CD28null T-cells, is linked to negative outcomes in several inflammatory conditions such as ANCA associated vasculitis (AAV), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), chronic kidney disease (CKD) as well as in cardiovascular disease and older individuals [112, 171, 173-175, 191, 287, 356]. Central to the rationale for the present study is the hypothesis that this is achieved, at least in part, by subclinical reactivation of the virus from latency resulting in a CMV mediated expansion of CD4+CD28null T-cells. The data presented in the previous chapters have shown that CD4+CD28null T-cells in AAV are largely CMV specific, Th-1, proinflammatory cells, that possess receptors which can target the endothelium and that the size of their expansion directly correlates with increased arterial stiffness, an established marker of cardiovascular risk. It is therefore important and clinically relevant to determine whether anti-CMV therapy has the potential to control possible subclinical CMV reactivation in latency and ameliorate these CMV induced changes on the immune system.

Utilising AAV as a model for inflammatory disease, the CANVAS proof of concept clinical trial (EudraCT 2012-001970-28, NCT01633476) presented here aimed to test the hypothesis that episodes of subclinical CMV reactivation in inflammatory conditions lead to an expansion of the CD4+CD28null T-cell compartment. To that end, the trial was designed to test whether a 6 month treatment with oral valaciclovir in CMV seropositive AAV patients in disease remission, can control subclinical CMV reactivation and whether by controlling subclinical CMV reactivation the size of the CD4+CD28null T-cell expansion can be ameliorated as well as whether this can lead to an improvement in other associated CMV driven changes to the immune system.

The trial protocol was approved by the relevant regulatory bodies and is outlined in Section 2.3. Patients were randomised to 2g QDS of oral valaciclovir (dose was reduced according to renal function, Table 2.4) or no additional treatment in an open label fashion for 6 months followed by an additional follow-up period of 6 months.

5.2 Recruitment and patient flow

The CONSORT flow diagram (Figure 5.1) shows the flow of patients within the clinical trial from assessment of eligibility to randomisation. In summary, 200 AAV patients were assessed for eligibility. Seventy-nine patients were initially deemed eligible for inclusion into the clinical trial. One of the main reasons for the lower number of eligible patients, compared to the estimated number of 125 prior to commencement

of the trial, was an increase in the use of annual or 6-monthly rituximab for maintenance of clinical remission that precluded inclusion into the trial as patients could not have had rituximab within the last 12 months (see exclusion criteria in Table 2.3). A substantial amendment was successfully submitted to the REC and MHRA to reduce the length of time from treatment with the T-cell depleting agent cyclophosphamide to inclusion in the trial, from 12 months to 6 months, as previous data in SLE had shown return of lymphocyte counts to pre-treatment values as early as 6 months following cyclophosphamide treatment [357]. An additional nine eligible patients were identified following this change.

Forty-nine patients declined to participate in the clinical trial although a number of those agreed to participate in the cross-sectional element of the study that involved a single visit (Chapters 3 and 4). The most common reason that patients did not want to participate was not wanting to take an extra tablet mainly due to apprehension of potential side effects and in particular fear of loss of kidney function as a result of the valaciclovir treatment. The second most common reason was the number of visits associated with the clinical trial. Thirty-nine patients were consented to participate in the clinical trial (44% of fully eligible patients). However, one patient had a family emergency shortly after consenting and was therefore not randomised as they were unable to participate. The remaining 38 patients were randomised to receive either valaciclovir or no additional therapy using the PC-CRTU independent telephone based randomisation system as described in Section 2.3.5.

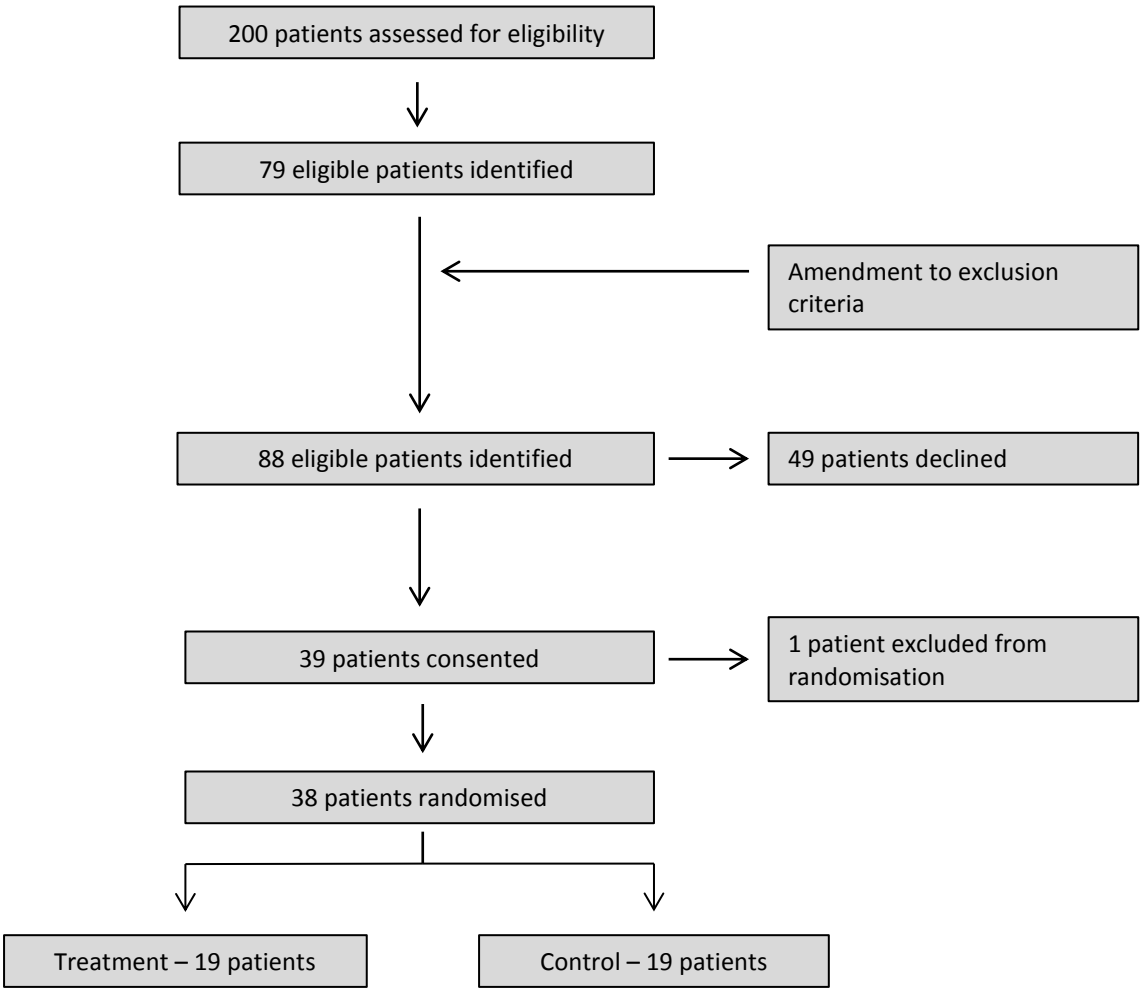

Figure 5.1 CONSORT flow diagram for the CANVAS clinical trial

5.3 Protocol compliance

There was good adherence to the study protocol during the course of the trial. There were no protocol breaches. Out of a total of 494 study visits only 27 visits were missed by study participants (5.5%). Two patients randomised to the control arm (CT022 and CT026) were only able to attend the baseline and month 6 visits. These two patients

were therefore not included in the analysis of the tertiary outcome (Section 5.7) and the change in CMV IgG titre exploratory outcome (Section 5.8.6) but were included in all other analyses.

The dose of valaciclovir was adjusted according to creatinine clearance. The median dose of valaciclovir prescribed was 6g / day (1.5g QDS) (range 3 – 8g / day; 1.5g BD – 2g QDS). Compliance with the prescribed trial medication was assessed in a random selection of 9 patients randomised to the treatment arm by counting the number of tablets returned at each month visit and comparing it to the number of tablets dispensed and the prescribed dose. The median compliance with prescribed trial medication in this selection of patients assessed was 94.4% (IQR 88.0 – 95.8) across the duration of the treatment period. This selection of patients included patients that might have had a temporary break in treatment.

All patients randomised to the treatment arm completed the study in full. However, one patient (CT035) elected to stop taking the tablet within 2 weeks of commencement whilst an additional patient (CT033) developed acute kidney injury, which led to discontinuation of the study drug within one month of commencement. Both patients completed subsequent trial visits fully although the study drug was not restarted. One patient (CT006) elected to stop the trial medication for 2 weeks during an episode of a chest infection at month 3 of the study. A further patient (CT031) had a 1-week break in treatment in month 2 due to a hospital admission unrelated to the

study drug and a 2-week break in treatment during month 4 associated with an episode of depression again unrelated to the study drug. All primary analyses were carried out using the intention to treat principle as pre-specified in the published clinical trial protocol [325].

5.4 Patient characteristics

All patients randomised to the clinical trial, in keeping with the eligibility criteria, were in disease remission with no documented relapses in the last 6 months. All patients had received high dose corticosteroids and cyclophosphamide or rituximab (with or without plasma exchange) for induction of remission, followed by low dose corticosteroids, azathioprine, mycophenolate mofetil (MMF) or methotrexate as maintenance of remission therapy. Three patients had discontinued immunosuppression at the time of commencing the trial (Table 5.1).

There were no significant differences between treatment and control groups in terms of age, gender, ethnicity, immunosuppressant medication or duration of disease (Table 5.1). Control patients had a higher proportion of CD4+CD28null T-cells at the pre-randomisation visit compared to patients randomised to treatment although this did not attain statistical significance. This discrepancy was noted at an interim Trial Steering Committee (TSC) meeting. Given the potential bias this may have caused at the end of the study, statistical advice given by the TSC statistical advisor suggested

the analysis plan be designed to assess proportionate rather than absolute value reduction for secondary, tertiary and exploratory outcomes.

Table 5.1 Characteristics of patients randomised to the clinical trial

	Total study group (n=38)	Treatment arm (n=19)	Control arm (n=19)	p value (treatment vs. control)
Age (years)	67.4 (10.2)	66.0 (11.5)	68.8 (8.8)	0.412
Gender (M:F)	25:13	12:7	13:6	0.732
Ethnicity (% Caucasian)	92.1	84.2	100.0	0.230
ANCA specificity (PR3:MPO)	27:10	12:6	15:4	0.476
Disease chronicity (months)*	71.5 (38.0 – 144.0)	87.0 (34.0 – 138.5)	70.0 (38.5 – 136.5)	0.930
Renal function eGFR (mL/min/1.73m^2)	56 (20)	53 (22)	59 (18)	0.339
Immunosuppression (% on steroids)	73.7	68.4	78.9	0.714
Immunosuppression (% on MMF)	26.3	26.3	26.3	1.000
Immunosuppression (% on azathioprine)	36.8	31.6	42.1	0.501
Immunosuppression (% on no treatment)	7.9	10.5	5.3	1.000
CD4+CD28- % at pre-randomisation visit*	13.5 (3.6 – 21.3)	10.9 (2.5 – 15.8)	19.1 (7.5 – 25.1)	0.102

Values presented as mean (SD) apart from * that is presented as median (IQR)

Eleven patients had their immunosuppression modified during the clinical trial period (Table 5.2). Such modifications were equally split between the two groups. The

majority of those instances related to a reduction or cessation of immunosuppression due to immunosuppressive treatment related complications such as infection. None of the participants had an episode of disease relapse during the trial.

Table 5.2 Modifications to immunosuppression during clinical trial period

ID	**Time point**	**Immunosuppressive treatment modification**	**Reason**
		Treatment Group	
CT031	Month 2	MMF paused for 2 weeks	Infection
CT021	Month 5 Month 9	Patient initiated temporary increase in prednisolone dose	Infection
CT005	Month 8	Azathioprine changed to MMF	To enable commencement of allopurinol for severe gout
CT006	Month 8	Azathioprine paused for 3 months	Shingles
CT035	Month 8	Temporary increase in prednisolone dose	Infection
CT005	Month 10	Temporary increase in prednisolone dose	Severe polyarticular gout
CT027	Month 10	Azathioprine stopped	Squamous cell carcinoma
		Control Group	
CT019	Month 2	Temporary course of prednisolone and MMF dose reduced	Infection
CT030	Month 3	Azathioprine dose reduced	Maintenance therapy dose reduction
CT028	Month 4	Azathioprine stopped	Renal mass – under investigation
CT026	Month 7	Temporary increase in prednisolone dose	Infection
CT034	Month 9	MMF dose reduced	Recurrent infections

MMF=Mycophenolate mofetil, ID=Clinical Trial Patient Identification

5.5 Primary Outcome

The primary outcome of the clinical trial was the proportion of patients with CMV reactivation in blood or urine as assessed by qPCR. All primary analyses were performed using the UHB Virology Laboratory validated cut-off of 200 CMV viral DNA copies / mL. Additional secondary analyses were performed with a cut-off of 20 viral copies / mL which is the lower limit of quantitation of the assay. A sensitivity analysis was performed on the proportion of reactivation episodes amongst all samples assayed. CMV reactivation episodes are shown in Table 5.3. The remainder of patients not shown on the table did not have any detectable CMV reactivation episodes for the duration of the clinical trial.

There were 12 reactivation episodes (>200 viral copies / mL) in 8 patients (21.1% of the study group) over the 12-month study period. In all of these reactivations episodes CMV DNA was detected only in the urine and not in plasma. An additional 27 reactivation episodes of minimal intensity (between 20 and 200 viral copies / mL) in total were observed in 15 patients (39.5% of the study group). CMV DNA was detected just in urine in 15 of these episodes, just in plasma in 10, and in both urine and plasma in 2 cases. All patients who had a reactivation of > 200 viral copies / mL had further episodes of minimal intensity reactivation. There was no correlation between CMV reactivation in the urine and renal function. All episodes were asymptomatic.

Table 5.3 CMV viral copy numbers per millilitre in clinical trial patients that reactivated CMV

ID	M0	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
						Treatment Arm							
CT003	28[p] ND[u]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	29[p] ND[u]	26[p] ND[u]
CT005	**624[u]** ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	27[p] ND[u]	ND[pu]	**315[u]** ND[p]	27[p] 46[u]	25[u] NS[p]	ND[pu]
CT014	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	20[p] ND[u]	ND[pu]	**3840[u]** ND[p]	ND[pu]
CT024	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	25[p] ND[u]
CT031	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	29[u] ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	**345[u]** ND[p]	NS[u] ND[p]
CT033	61[u] ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[u] NS[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]
CT035	ND[pu]	103[u] ND[p]	ND[pu]	38[u] ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]
CT038	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	164[u] ND[p]	ND[pu]	ND[pu]
						Control Arm							
CT002	**229[u]** ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	75[u] ND[p]
CT009	ND[pu]	ND[pu]	ND[pu]	81[u] ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	102[u] ND[p]	ND[pu]	ND[pu]	ND[pu]
CT012	ND[pu]	27[p] 195[u]	**3382[u]** ND[p]	ND[pu]	ND[pu]	21[p] ND[u]	ND[pu]	ND[pu]	81[u] ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]
CT016	ND[pu]	ND[pu]	ND[pu]	ND[pu]	24[u] ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]
CT019	ND[pu]	ND[pu]	63[p] ND[u]	ND[pu]	32[u] ND[p]	**1106[u]** ND[p]	27[u] ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	47[p]
CT020	ND[pu]	ND[pu]	ND[pu]	44[u] ND[p]	ND[pu]	**211[u]** ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]
CT037	ND[pu]	**1448[u]** ND[p]	**211[u]** ND[p]	23[u] ND[p]	**831[u]** ND[p]	ND[pu]	ND[pu]	**271[u]** ND[p]	ND[pu]	ND[pu]	ND[pu]	ND[pu]	ND[pu]

Key:

[p] plasma, [u] urine, ND=not detected, NS=no sample available,

M0=baseline visit, M1=month 1

ID=Clinical Trial Patient Identification

Reactivation episodes > 200 copies / mL are in bold.

Primary analysis of CMV reactivation episodes (> 200 viral copies / mL) between month 1 and month 6 (i.e. the duration of treatment), was consistent with valaciclovir treatment successfully blocking CMV reactivation completely in the treatment group with 4 patients reactivating in the control group (21.1%) and none in the treatment group (p=0.037). During the 6 month follow up period after cessation of treatment, patients in the treatment group reactivated at similar rates to the control group and by the end of the 12-month period there was no difference in the overall proportion of patients with CMV reactivation in the control group (21.1%) versus the treatment group (15.8%)(p=0.449) (Figure 5.2).

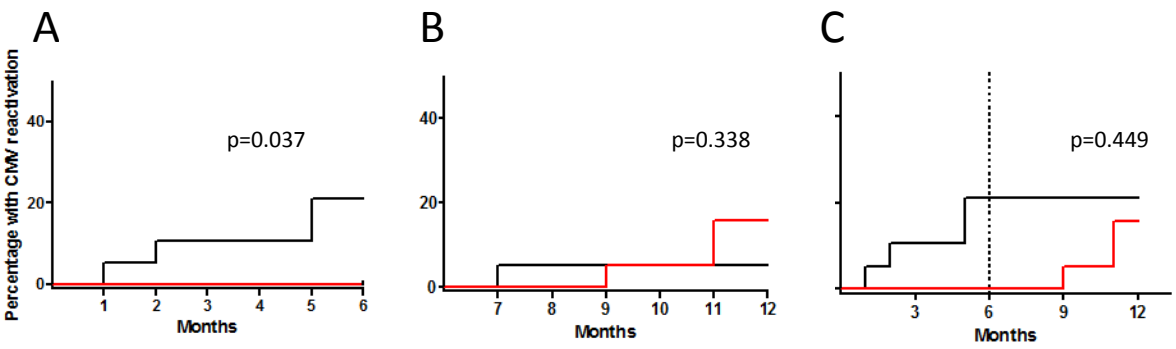

Figure 5.2 Kaplan-Meier curve analysis of the primary outcome showing time to reactivation (>200 viral copies / mL; primary analysis) in the treatment (n=19) (red line) and control (n=19) (black line) groups

Curves were compared using the Gehan-Breslow-Wilcoxon statistic. Panels A, B, C show the treatment period (Month 1 – Month 6), follow-up period after cessation of treatment only (Month 7 – Month 12) and overall study period (Month 1 – Month 12), respectively. The gridline in panel C indicates the end of treatment.

A secondary analysis of the primary outcome was also performed in order to consider those CMV reactivation episodes of minimal intensity (20 – 200 viral copies / mL). On an intention to treat analysis, more control patients reactivated (>20 viral copies / mL) compared to treatment patients during the treatment period (Month 1 – Month 6) (control 31.6% vs. treatment 10.5%; p=0.110), although this difference did not reach statistical significance. However, two patients in the treatment group (CT035 and CT033) stopped taking the drug at 2 weeks and at 1 month respectively as mentioned earlier. Indeed, two out of the three reactivation episodes in the treatment group during the treatment period occurred in patient CT035 following cessation of the study drug at 2 weeks. The third episode occurred in patient CT031 who had a 2-week break in treatment during month 4 and reactivated at the month 5 time point. On a per protocol analysis that did not include the 2 patients that had stopped treatment by month 1, there was a statistically significant difference in the proportion of patients reactivating CMV (>20 viral copies / mL) between the two groups during the treatment period (control 31.6% vs. treatment 5.3%; p=0.041). In addition, the only episode of CMV reactivation in the treatment group occurred in patient CT031 following a 2 week break in treatment as described above (Figure 5.3). As with the primary analysis, following the end of treatment at month 6, both groups reactivated CMV at similar rates.

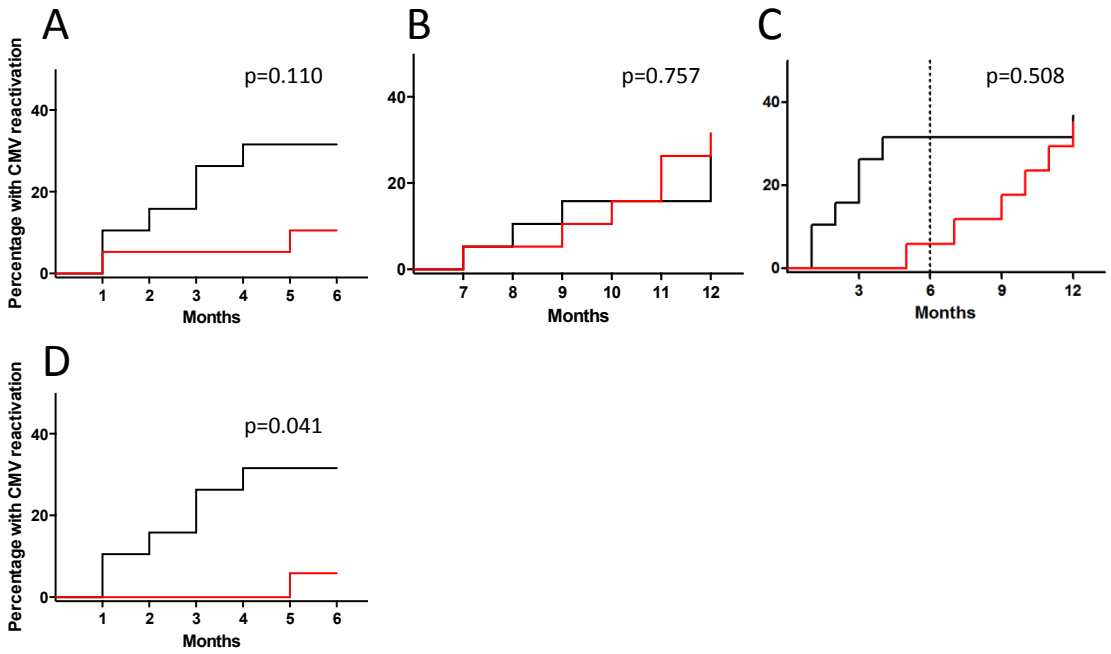

Figure 5.3 Kaplan-Meier curve analysis of the primary outcome showing time to reactivation (>20 viral copies / mL; secondary analysis) in the treatment (n=19) (red line) and control (n=19) (black line) groups

Curves were compared using the Gehan-Breslow-Wilcoxon statistic. Panels A, B, C show the treatment period (Month 1 – Month 6), follow-up period after cessation of treatment only (Month 7 – Month 12) and overall study period (Month 1 – Month 12) respectively (all intention to treat). The gridline in panel C indicates the end of treatment. Panel D shows the results of the per protocol analysis during the treatment period (Month 1 – Month 6).

A sensitivity analysis was also performed to consider the proportion of samples that were positive for CMV reactivation amongst all plasma and urine samples assayed during the treatment period. Contingency tables were constructed and the treatment

group was compared to the control group using the Fisher's exact test (Table 5.4). Missing samples were taken into account by excluding them from the denominator. Utilising the 200 viral copies / mL threshold, there were 6 reactivation instances out of a possible of 205 in the control group and 0 instances out of a possible of 227 in the treatment group during the treatment period (p=0.011). Considering all reactivation episodes greater than 20 viral copies / mL, the numbers were 16 out of 205 and 3 out of 227 for the control and treatment groups respectively (p=0.002). This analysis was performed on an intention to treat basis and included all patients randomised to the treatment arm. Finally, during the treatment period, taking into account all episodes >20 viral copies / mL, the median CMV viral DNA copy number was 741.5 copies / mL (IQR: 66.8 – 2791.0) in the control group compared to just 85.0 copies / mL (IQR: 29.0 – 141.0) in the treated patients. As mentioned earlier, it is important to note that the 3 reactivation episodes of minimal intensity (20 – 200 viral copies / mL) that occurred in the treatment group during the treatment period all occurred either after cessation of treatment or following a break in treatment. Taken together this data shows that valaciclovir treatment successfully blocked CMV reactivation during the treatment period. Following cessation of therapy, CMV reactivation resumed at the same rate as that seen in the control arm.

Table 5.4 Contingency table showing number of reactivation episodes during the treatment period

	Treatment	Control
Number of samples with > 200 viral copies / mL	0	6
Number of samples with > 20 viral copies / mL	3	16
Total number of samples assayed (plasma and urine)	227	205
Number of missing samples	1	23

5.6 Secondary Outcomes

Given the central role of CD4+CD28null T-cells as mediators of CMV driven inflammation and in order to test the hypothesis that subclinical CMV reactivation drives the expansion of CD4+CD28null T-cells, one of the main secondary outcomes of the clinical trial was change in the proportion of CD4+CD28null T-cells from baseline to 6 months. Other pre-specified secondary outcomes were change in the concentration of soluble markers of inflammation (CRP, IFN-γ, TNF-α, IL-2, IL-6, IL-10, IL-17) from baseline to 6 months and safety.

5.6.1 Secondary Outcome – Change in the proportion of CD4+CD28null T-cells from baseline to 6 months

The CD4+CD28null T-cell percentage within the CD4 compartment was measured via flow cytometry after staining whole blood with fluorochrome labelled monoclonal antibodies for CD3, CD4 and CD28 as detailed in Section 2.6.2. A fluorescence minus one (FMO) control for CD28 staining was run with every experimental sample in order to aid gating. The gating strategy has been discussed in Chapter 3 (Figure 3.1).

The assay was validated for use in the clinical trial as detailed in Section 2.7. An aliquot of Cytofix CD4 positive control with a known CD3+CD4+ expression was assayed with each experimental assay run to provide quality assurance and verify that each assay was completed successfully.

The absolute values of CD4+CD28null T-cell percentages at the start (baseline) and at the end (month 6) of the treatment period for treated and control patients are shown in Figure 5.5. These were analysed using paired ratio t tests as described in Section 2.9.1, in order to test for statistically significant trends in the ratio of CD4+CD28null T-cell percentage between month 6 and baseline in the treatment and control groups. Data is reported as geometric mean of ratios with 95% confidence intervals (CI), where a value of less than 1 denotes a reduction in the ratio of the percentage of CD4+CD28null T-cells (month 6 to baseline) and a value greater than 1 an increase.

There was a statistically significant reduction in the CD4+CD28null T-cell percentage month 6 to baseline ratio in treated patients, of 23.0 (geometric mean of ratios 0.770, 95% CI [0.611 - 0.970], p=0.029). In contrast, there was no statistically significant change in the CD4+CD28null T-cell percentage ratio in the control patients (0.946 [0.814 - 1.110], p=0.449) indicating that blocking subclinical CMV reactivation in the treated patients was associated with a reduction in CD4+CD28null T-cell proportion.

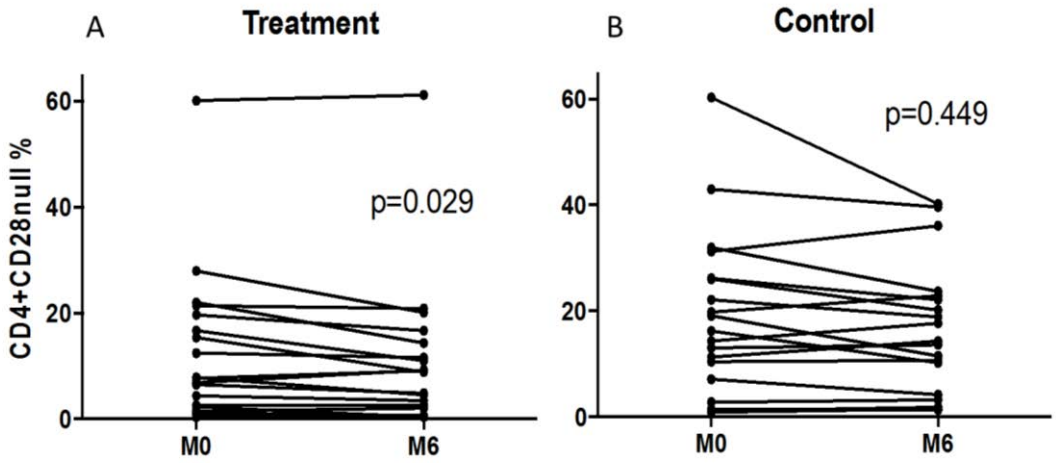

Figure 5.5 Secondary outcome – change in CD4+CD28null T-cell percentage during treatment period

Panels A and B show absolute values of CD4+CD28null T-cell percentages at baseline (M0) and month 6 (M6) for treatment (n=19) and control (n=19) patients respectively. There was a significant proportionate reduction in CD4+CD28null T-cell percentage at the end of the treatment period in treated patients but not controls.

5.6.2 Secondary Outcome – Change in concentration of soluble markers of inflammation from baseline to 6 months

In order to determine whether blocking subclinical CMV reactivation also led to an improvement in soluble markers of inflammation, plasma CRP, IFN-γ, TNF-α, IL-2, IL-6, IL-10, and IL-17 were measured using Luminex technology at the start and end of treatment as detailed in Section 2.6.3. Paired ratio t tests were used to detect statistically significant trends between the baseline and month 6 measurements in the treatment and control groups as previously described.

There was a statistically significant reduction in the ratio of month 6 to baseline concentration of IL-2 in the treated patients, of 81.6% (geometric mean of ratios 0.184, 95% CI [0.049 - 0.693], p=0.015), whereas no statistically significant change was observed in the control patients (0.831 [0.522 - 1.323], p=0.414). There was also a reduction in the ratio of month 6 to baseline concentration of IFN-γ in the treated patients, of 81.6% (0.183 [0.033 - 1.011], p=0.051), whereas again no change was seen in control patients (1.166 [0.657 - 2.071], p=0.580) (Figure 5.6). There were no statistically significant differences in the ratios of the month 6 to baseline concentration values for TNF-α, IL-6, IL-10, IL-17 or CRP in either of the groups (Figure 5.7). Summary data of month 6 to baseline ratios for all plasma markers of inflammation measured as part of the secondary outcome are shown in Table 5.5.

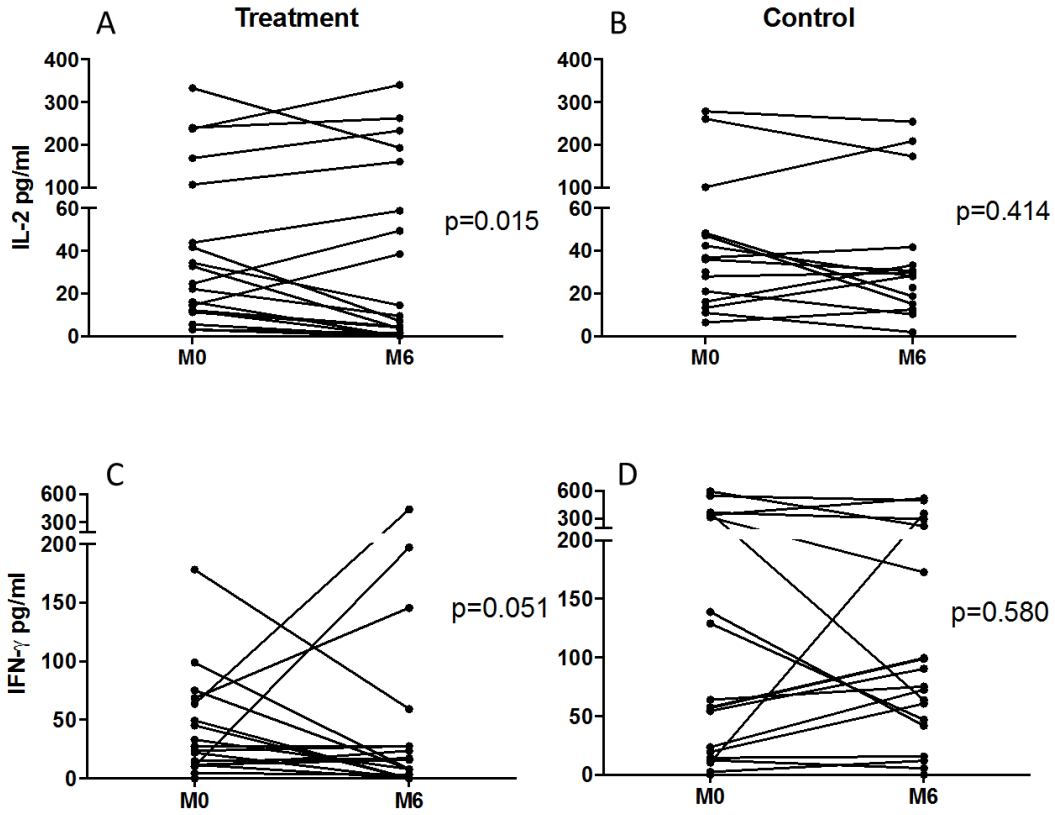

Figure 5.6 Secondary outcome – change in plasma concentration of markers of inflammation

Panels A and B show absolute values of IL-2 and panels C and D of IFN-γ concentration at baseline (M0) and month 6 (M6) for treatment (n=19) and control (n=19) patients respectively. At the end of the treatment period there was a statistically significant proportionate reduction in the plasma level of IL-2, as well as a trend towards a reduction in the plasma level of IFN-γ, in the treated patients but not controls.

Table 5.5 Results of paired ratio t tests for soluble markers of inflammation assayed for the clinical trial secondary outcome

	Treatment			Control		
Marker	Geometric mean of ratios (M6:M0)	95% CI	p value	Geometric mean of ratios (M6:M0)	95% CI	p value
IL-2	0.184	[0.049 - 0.693]	0.015	0.831	[0.522 - 1.323]	0.414
IFN-γ	0.183	[0.033 - 1.011]	0.051	1.166	[0.657 - 2.071]	0.580
TNF-α	0.612	[0.343 - 1.093]	0.092	0.859	[0.501 - 1.472]	0.560
IL-6	0.478	[0.187 - 1.220]	0.115	0.914	[0.636 - 1.314]	0.610
IL-10	0.682	[0.344 - 1.354]	0.256	0.811	[0.541 - 1.214]	0.290
IL-17	0.339	[0.087 - 1.327]	0.113	0.405	[0.101 - 1.622]	0.188
CRP	1.304	[0.632 – 2.691]	0.451	0.887	[0.465 – 1.690]	0.700

M6 = month 6, M0 = baseline, CI = confidence intervals

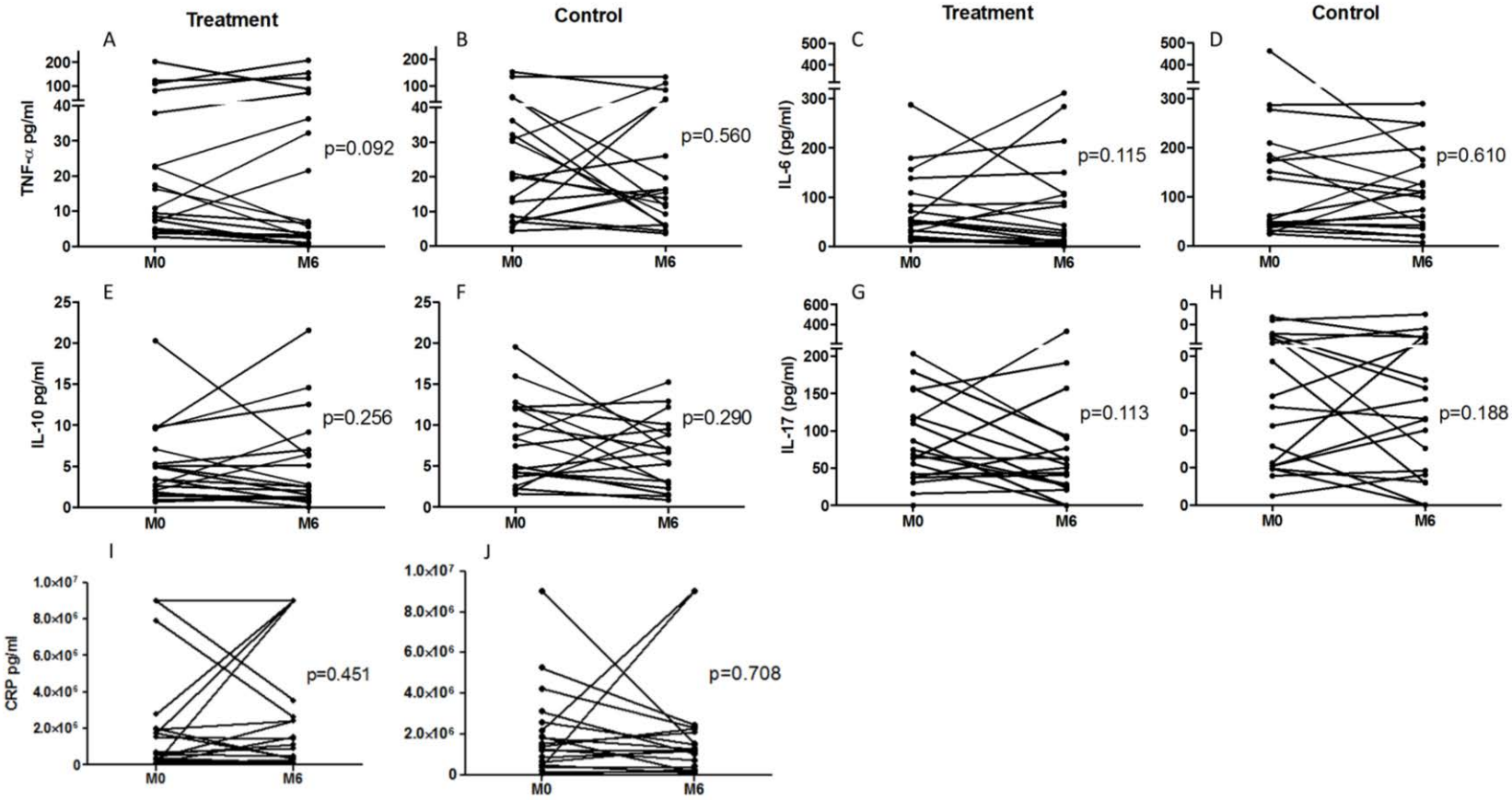

Figure 5.7 Secondary outcome – change in plasma concentration of markers of inflammation. Panels show absolute values for concentration of plasma markers at baseline (M0) and month 6 (M6). Panels A, C, E, G and I show values for TNF-α, IL-6, IL-10, IL-17 and CRP in the treated patients whilst panels B, D, F, H and J show absolute values for the concentration of the same markers at M0 and M6 for the control patients.

5.6.3 Safety

5.6.3.1 Adverse events

Adverse events (AE) were documented for both treatment and control patients for the duration of the study irrespective of assessed relatedness at the time of reporting (Table 5.6). Overall, the study drug was well tolerated. There was no difference in the number of observed episodes of infection during the clinical trial period between treated and control patients. The majority of infections were either lower or upper respiratory tract infections. In addition, there was one episode of shingles in a control patient who received a one week course of aciclovir by their GP during month 2 of the study. There was also an episode of shingles in a treatment patient that developed 2 months following cessation of the study drug. This was also treated with a course of aciclovir treatment by the GP.

There were significantly more gastrointestinal tract (GI) related AEs reported in the treatment group compared to control patients. This is in keeping with the known side-effect profile of valaciclovir. The majority of GI related AEs were episodes of diarrhoea, nausea and vomiting, and to a lesser extent abdominal pain, dry mouth and weight loss. All reported GI related events were mild in severity and short lived and did not lead to discontinuation of the study drug by the participants or the research team. In addition, 14 out of the 36 GI related observed AEs in the treatment group occurred in 2 patients with a known long-standing history of chronic diarrhoea and were assessed as not being related to the study medication at the time of reporting.

Table 5.6 Adverse events reported during the clinical trial period

	Total study group (n=38)	Treatment arm (n=19)	Control arm (n=19)	p value (treatment vs. control)
Total number of infections	84	43	41	0.827
Total number of GI related AEs	49	36	13	0.002
Total number of musculoskeletal AEs	36	23	13	0.100
Number of patients with new or worsening respiratory AEs	22	11	11	1.000
Number of patients with new or worsening cardiac AEs	5	3	2	1.000
Number of patients with new or worsening neuropsychiatric AEs	14	8	6	0.501
Number of patients experiencing bleeding or bruising	9	4	5	1.000
Number of patients reporting new or worsening lethargy	12	6	6	1.000
Number of patients with new ocular / visual AEs	6	3	3	1.000
Number of patients with new ENT related AEs	3	2	1	1.000
Episodes of acute kidney injury	3	2	1	1.000
Episodes of anaemia	3	2	1	1.000
Liver dysfunction episodes	1	1	0	1.000

GI = gastrointestinal tract

One patient elected to stop the study drug after 2 weeks of treatment due to a perceived increase in long-standing breathlessness. Although this episode was assessed as not related to the study drug, the study medication was not restarted as per the patient's wishes. There was no difference in the frequency of respiratory AEs between treatment and control patients.

Other AEs were equally split between the treatment and control patients (Table 6.6). Of note, a squamous cell carcinoma was diagnosed in a treatment patient and a renal mass was identified in a control patient during the study period.

There were 3 episodes of acute kidney injury (AKI) in 3 patients during the study period. One of those occurred in a treatment patient several months after cessation of the study drug in the context of a hospital admission for severe polyarticular gout and was assessed as not related to the study drug. Another AKI episode occurred in a control patient in the context of a hospital admission associated with pulmonary emboli. A further episode of AKI occurred in a treatment patient within 1 month of commencing the study drug. Creatinine rose to a value of 176 μmol/L (baseline 70 μmol/L). This was associated with symptoms of neurotoxicity (drowsiness, unsteadiness and mild dysarthria) and anaemia in keeping with valaciclovir toxicity and the drug's known side effect profile. The study drug was promptly stopped on review at the month 1 visit. All symptoms resolved following cessation of valaciclovir and renal function returned to baseline over the course of 2 weeks. The episode was managed on an outpatient basis and the study drug was not restarted.

One episode of mild derangement in liver function occurred in a patient randomised to the treatment arm during the treatment period. Alanine transaminase (ALT) rose to a maximum value of 55 IU/L. This occurred following commencement of cinnarizine for long-standing inner ear dysfunction. The ALT level returned to normal following

discontinuation of cinnarizine without stopping the study drug. The transient mild ALT rise was assessed as not related to the study medication.

5.6.3.2 Transient rise in mean cell volume

An incidental macrocytosis was consistently observed amongst the patients that received the study drug. Mean cell volume (MCV) gradually increased in valaciclovir treated patients, reaching a plateau at month 5 (Figure 5.8). Following cessation of the study drug at month 6, MCV gradually reduced and reached normal values by the end of the study period. This pattern was not observed in the control patients. The macrocytosis associated with the study drug was asymptomatic and not accompanied by any change in haemoglobin or other full blood count indices. Upon recognition of this AE in the first few patients, vitamin B12, folic acid and thyroid function tests were routinely checked in all treatment patients that developed a macrocytosis. There was no associated thyroid dysfunction, vitamin B12 or folate deficiency. There was an inverse relationship between renal function and peak MCV (Figure 5.8, Panel B), although this observation was not statistically significant.

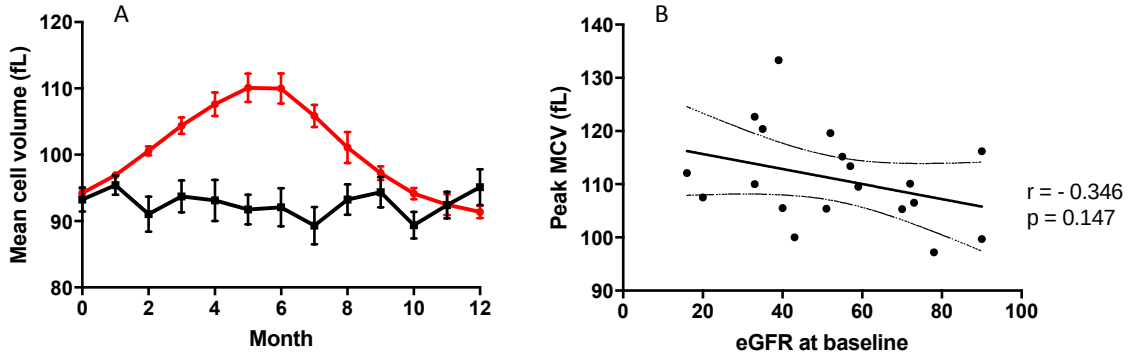

Figure 5.8 Transient macrocytosis in the treatment group

Panel A shows mean MCV (mean cell volume) in treated (n=19) (red) and control (n=17) (black) patients. Panel B shows a scatter plot of eGFR versus peak MCV observed in treated patients (linear regression line with 95% CI is shown).

5.6.3.3 Serious adverse events

A total of 12 serious adverse events (SAEs) were reported during the study period (see also DSUR in Appendix 9). All SAEs were assessed as not related to the study drug and SAEs were evenly split between the treatment and control groups (Table 5.7). One treatment patient had a total of 5 SAEs due to hospital admissions for recurrent infective exacerbations of bronchiectasis in keeping with their known past medical history and background frequency of infective exacerbations of bronchiectasis.

Table 5.7 Serious adverse events reported during the clinical trial period

	Total study group (n=38)	Treatment arm (n=19)	Control arm (n=19)
Respiratory infection	7	5	2
Gastrointestinal tract infection	1	0	1
Arthritis	1	1	0
Arrhythmia	1	0	1
Epistaxis	1	0	1
Pulmonary emboli	1	0	1

5.7 Tertiary Outcome – Persistence of valaciclovir effect on the proportion of CD4+CD28null T-cells

The persistence of the effect of blocking subclinical CMV reactivation on CD4+CD28null T-cell proportions was assessed by comparing the CD4+CD28null T-cell percentage within the CD4 compartment at 6 months and 12 months for treatment and control groups. Data was transformed and analysed using paired ratio t tests as described above. No statistically significant differences were detected in the month 12 to month 6 ratios of CD4+CD28null T-cell percentage in either of the groups (Treatment: geometric mean of ratios 0.893, 95% CI [0.682 - 1.171], p=0.393; Control: 1.146 [0.930 - 1.412], p=0.186).

In order to consider change in CD4+CD28null T-cell percentage across the duration of the study, a repeated measures ANOVA analysis was carried out with Dunnett's multiple comparison test as a post-hoc analysis, comparing baseline to month 6 and month 6 to month 12 CD4+CD28null T-cell percentage ratios. The absolute CD4+CD28null percentage values are shown in Figure 6.9 and the summary data in Table 5.8. The only statistically significant comparison was a reduction in the CD4+CD28null T-cell percentage ratio in the treatment group between baseline and month 6 (treatment period) in keeping with the findings already presented for the secondary outcome and indicating that blocking subclinical CMV reactivation with valaciclovir led to a sustained reduction in the proportion of CD4+CD28null T-cells in the treated patients.

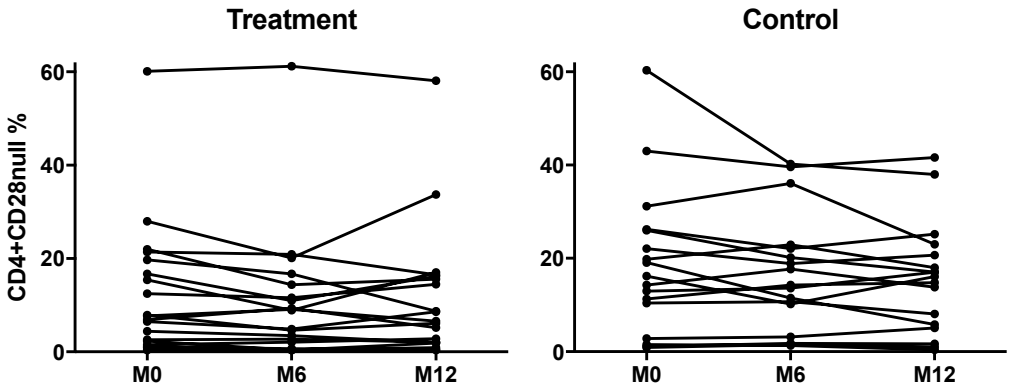

Figure 5.9 Change in CD4+CD28null T-cell proportion across the study period
Left and right panels show absolute values of CD4+CD28null T-cell percentages at baseline (M0), month 6 (M6) and month 12 (M12) for treatment (n=19) and control (n=17) patients respectively.

Table 5.8 Repeated measures ANOVA analysis for CD4+CD28null T-cell percentage change across study period

	Treatment			Control		
	Geometric mean of ratios	95% CI	Adjusted p value	Geometric mean of ratios	95% CI	Adjusted p value
Overall			0.065			0.267
M6:M0	0.770	[0.599 - 0.990]	0.041	0.987	[0.827 - 1.177]	0.975
M12:M6	0.893	[0.695 - 1.149]	0.489	1.146	[0.902 - 1.455]	0.309

Adjusted p values based on Dunnett's post-hoc multiple comparison test

5.8 Exploratory Outcomes

In order to determine whether blocking CMV reactivation with valaciclovir led to an improvement in other associated CMV driven changes to the immune system exploratory outcomes included change in CD8CD28null T-cells, CD4:CD8 ratio, soluble markers of endothelial dysfunction and CMV IgG titre. Change in the absolute count of CD4+CD28null, CD8CD28null and IFN-γ producing CD4+CD28null T-cells following overnight stimulation with CMV lysate was also assessed.

5.8.1 Change in CD4+CD28null T-cell counts from baseline to 6 months

In keeping with the observed reduction in the proportion of CD4+CD28null T-cells in the treatment arm (secondary outcome), there was a reduction in the month 6 to

baseline ratio of the CD4+CD28null cell count of 27.0% in the treated patients (geometric mean of ratios 0.730, 95% CI [0.574 - 0.929], p=0.013), whereas no change was observed in the control patients (0.934 [0.7504 - 1.163], p=0.523) (Figure 5.10).

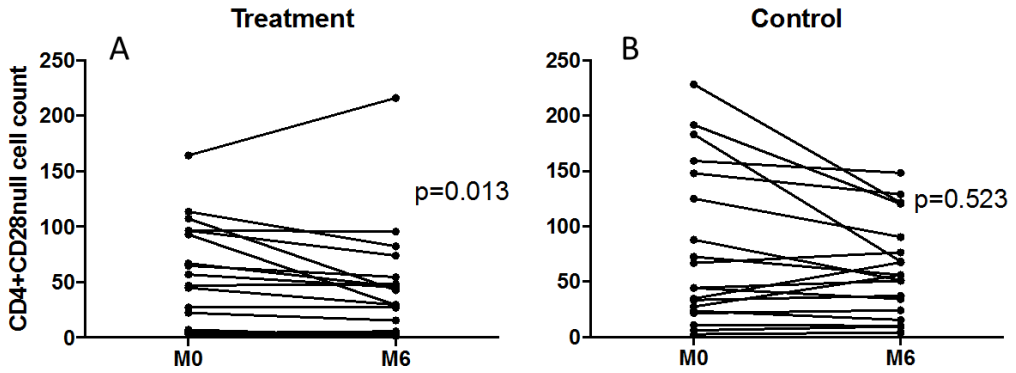

Figure 5.10 Exploratory outcome – change in CD4+CD28null T-cell count during treatment period

Panels A and B show absolute values of CD4+CD28null T-cell counts at baseline (M0) and month 6 (M6) for treatment (n=19) and control (n=19) patients respectively. There was a statistically significant proportionate reduction in the absolute CD4+CD28null T-cell count in treated patients but not in controls.

5.8.2 Change in CD8CD28null T-cell percentage and absolute count from baseline to 6 months

There was no significant change in the CD8CD28null T-cell percentage month 6 to baseline ratio in either of the two groups (Treatment: 0.921 [0.826 - 1.027], p=0.131;

Control: 0.984 [0.927 - 1.046], p=0.591) (Figure 5.11). There was however a statistically significant reduction in the CD8CD28null cell count ratio in the treatment group of 20.7% (0.793 [0.642 - 0.979], p=0.033) with no observed change in control patients (0.987 [0.786 - 1.238], p=0.901) (Figure 5.11).

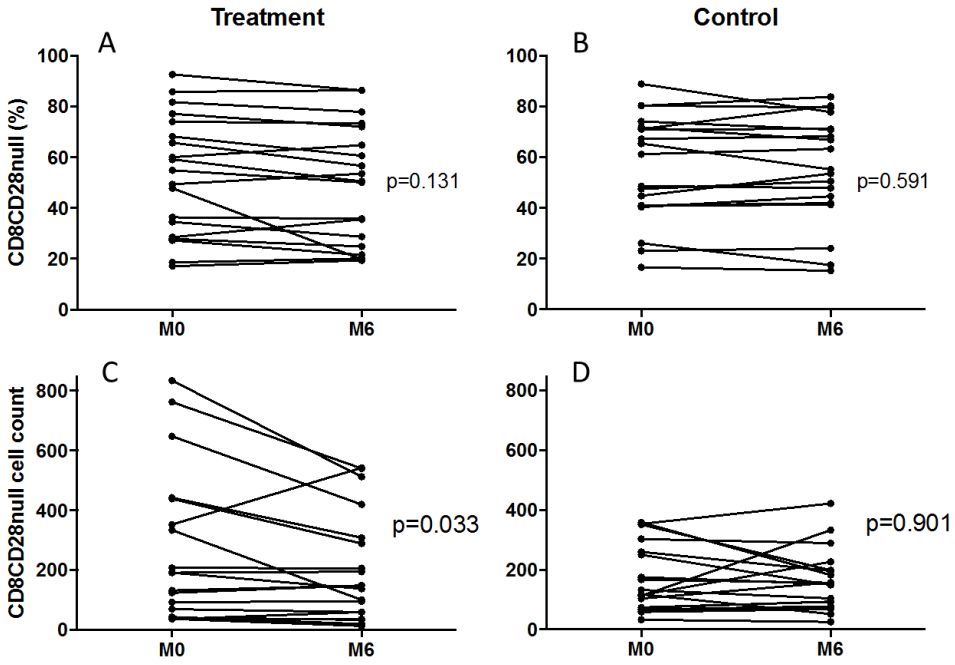

Figure 5.11 Exploratory outcome – change in CD8CD28null T-cell proportion and absolute count during treatment period

Panels A and B show absolute values of CD8CD28null percentages and panels C and D absolute values of CD8CD28null cell counts at baseline (M0) and month 6 (M6) for treatment (n=19) and control (n=19) patients respectively. There was no change in the percentage of CD8CD28null T-cells in either treated or control patients. In treated patients there was a proportionate reduction in the absolute count of CD8CD28null T-cells across the treatment period that was not observed in controls.

5.8.3 Change in the CD4:CD8 ratio from baseline to 6 months

An inverted CD4:CD8 ratio is one of the characteristics of the 'immune risk profile' (IRP) [288, 289, 291]. Given the fact that CMV is implicated in the development of the IRP [294] and the CD4+CD28null T-cell percentage was found to be associated with a reduced CD4:CD8 ratio in AAV patients (Chapter 3, Figure 3.2), change in the CD4:CD8 ratio across the treatment period was assessed. Blocking subclinical CMV reactivation with valaciclovir was associated with an improvement (i.e. an increase) in the CD4:CD8 ratio in the treated patients of 0.256 (95% CI 0.001 – 0.511, p=0.050) with no change observed in control patients (- 0.162 [- 0.566 – 0.242], p=0.411) (Figure 5.12).

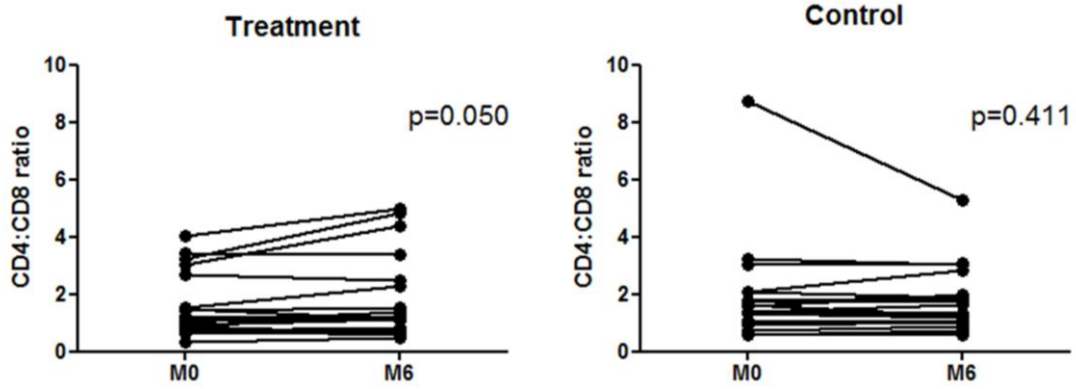

Figure 5.12 Change in CD4:CD8 ratio during treatment period

At the end of the 6 month treatment period there was an increase in the CD4:CD8 ratio corresponding to an improvement in this marker of the IRP in the treated (n=19) patients whilst no change was observed in controls (n=19).

5.8.4 Change in CD4+CD28null CMV specific T-cells from baseline to 6 months

Analysis of CD4+CD28null T-cells capable of producing IFN-γ following overnight stimulation with CMV lysate, revealed a statistically significant reduction in the CD4+CD28null IFN-γ+ percentage ratio between month 6 and baseline of 25.3% as well as a reduction in the CD4+CD28null IFN-γ cell count ratio of 45.0% in the treatment group (geometric mean of ratios 0.747 and 0.550, 95% CIs [0.565 - 0.989 and 0.358 - 0.856], p value 0.043 and 0.010). There was no significant change in the control patients (1.122 and 1.016, 95% CI [0.755 - 1.669] and [0.605 - 1.707], p value 0.548 and 0.949) (Figure 5.13). This was in agreement with the observed reduction in overall CD4+CD28null T-cell in the treated patients (secondary outcome) and indicated that blocking CMV reactivation with valaciclovir in the treated patients led to a targeted reduction in CMV specific CD4+CD28null T-cells.

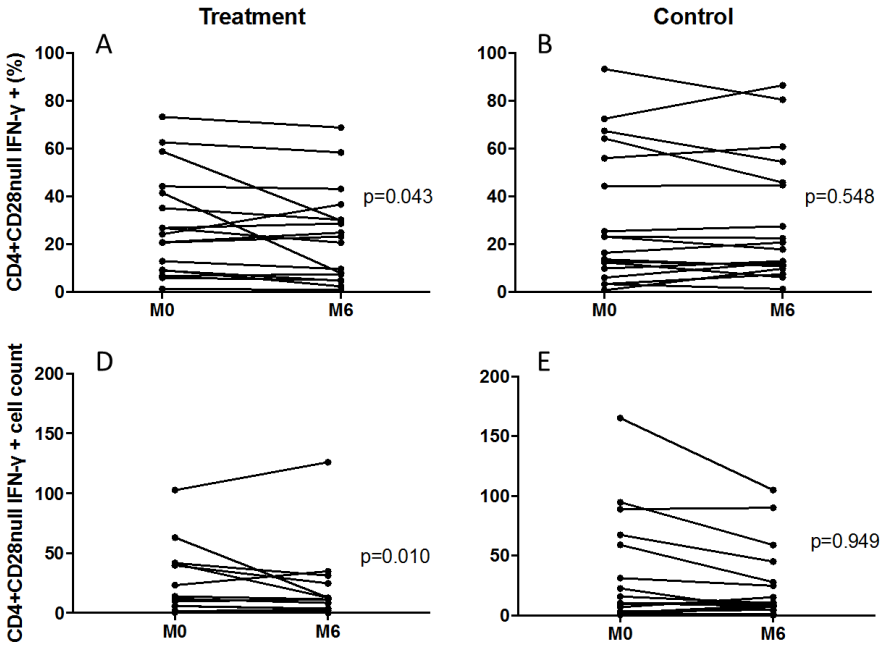

Figure 5.13 Exploratory outcome – change in CMV specific CD4+CD28null T-cell proportion and absolute count during treatment period

Panels A and B show absolute values of CD4+CD28null IFN-γ+ percentages and panels D and E absolute values of CD4+CD28null IFN-γ+ cell counts following overnight stimulation with CMV lysate at baseline (M0) and month 6 (M6) for treatment (n=19) and control (n=19) patients respectively. There was a proportionate reduction in both CD4+CD28null IFN-γ percentage and absolute count across the treatment period in the treated patients but not in controls.

5.8.5 Change in soluble markers of endothelial damage from baseline to 6 months

Soluble markers of endothelial dysfunction (RANTES, sVCAM-1, sICAM-1, P-selectin, E-selectin, Fractalkine, IP-10, MCP-1 and IL-12) were measured using Luminex technology at the start and end of treatment as detailed in Section 2.6.3 in order to determine whether blockage of CMV reactivation with valaciclovir would have an effect on the levels of these markers. Paired ratio t tests were used to detect statistically significant trends between the baseline and month 6 measurements in the treatment and control groups.

The only statistically significant change observed was in the level of P-selectin that decreased by 15% ($p=0.046$) in the treated patients whereas no statistically significant difference was observed in controls (Figure 5.14) (Table 5.9). There was no statistically significant change in the ratio of month 6 to baseline concentration of RANTES, sVCAM-1, sICAM-1, E-selectin, Fractalkine, IP-10, MCP-1 or IL-12 in either of the groups (Figure 5.15 and Table 5.9).

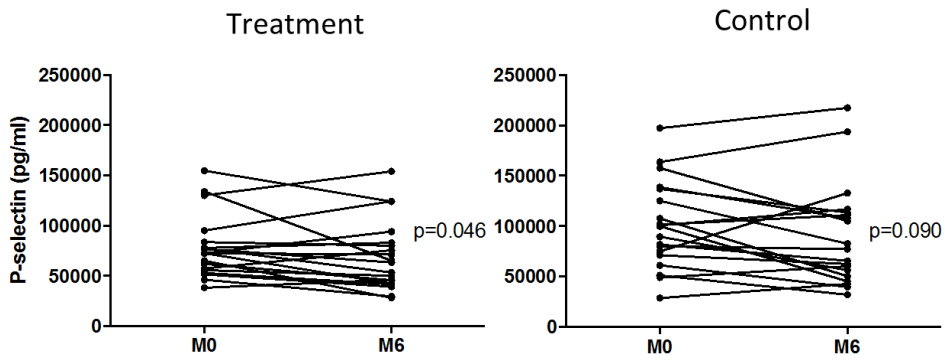

Figure 5.14 Exploratory outcome – Change plasma concentration of markers of endothelial dysfunction (P-selectin)

Absolute values are shown for treatment (n=19) and control (n=19) patients at baseline (M0) and month 6 (M6). There was a statistically significant proportionate reduction in the levels of P-selectin in the treated patients but not in controls.

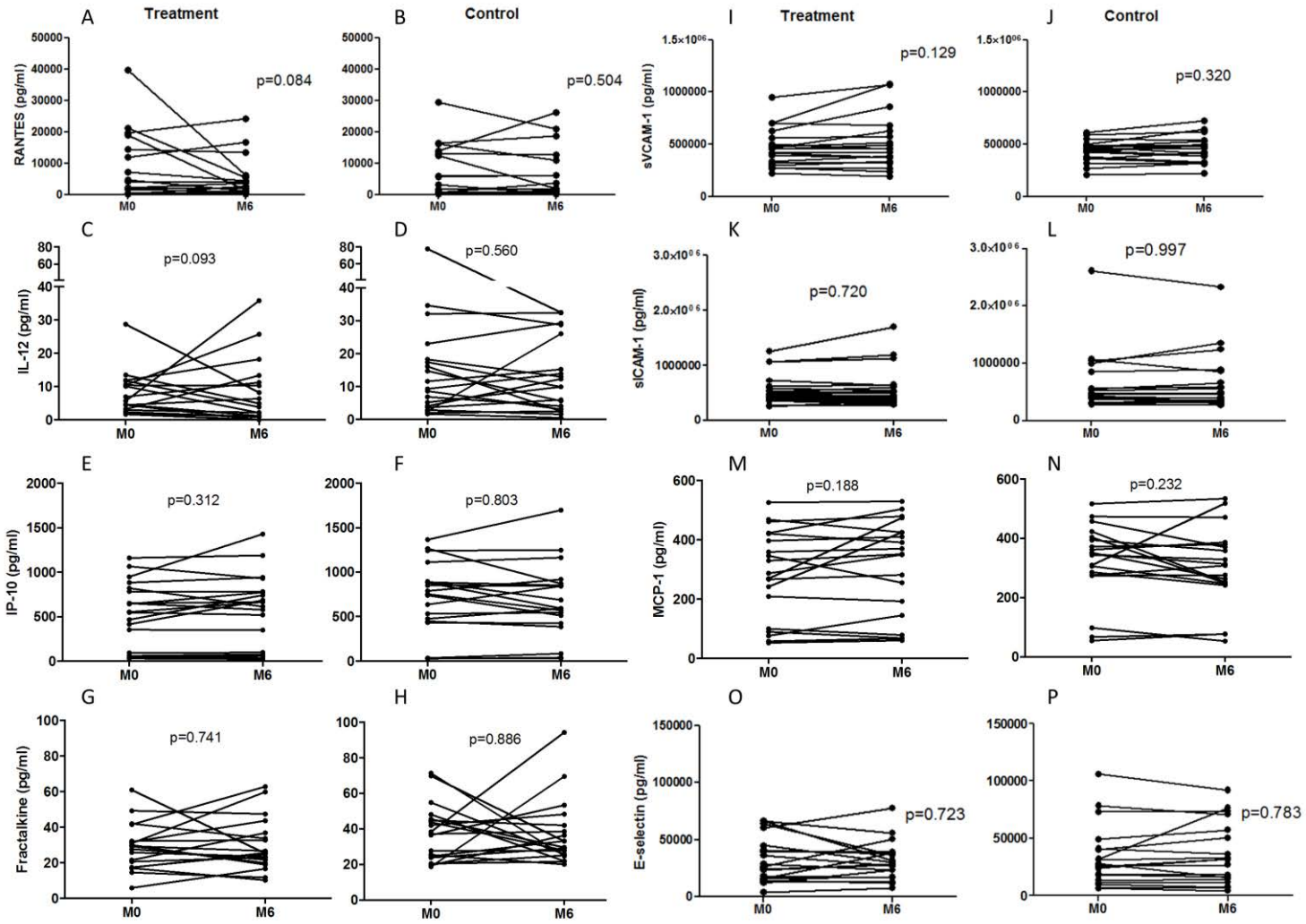

Figure 5.15 Exploratory outcome – change in plasma concentration of markers of endothelial dysfunction

Panels A, C, E, G, I, K, M and O show absolute values for concentration of RANTES, IL-12, IP-10, fractalkine, sVCAM-1, sICAM-1, MCP-1 and E-selectin respectively for baseline (M0) and month 6 (M6) in treated patients. Panels B, D, F, H, J, L, N and P show values for control patients.

Table 5.9 Results of paired ratio t tests for soluble markers of endothelial dysfunction

	Treatment			Control		
Marker	Geometric mean of ratios (M6:M0)	95% CI	p value	Geometric mean of ratios (M6:M0)	95% CI	p value
RANTES	0.602	[0.336 - 1.078]	0.084	0.832	[0.472 - 1.467]	0.504
P-selectin	0.850	[0.726 - 0.996]	0.046	0.859	[0.718 - 1.027]	0.090
E-selectin	1.037	[0.839 - 1.281]	0.723	1.019	[0.884 - 1.176]	0.783
sVCAM-1	1.058	[0.942 - 1.141]	0.129	1.030	[0.969 - 1.095]	0.320
sICAM-1	1.012	[0.945 - 1.084]	0.720	1.000	[0.924 - 1.082]	0.997
Fractalkine	1.036	[0.829 - 1.295]	0.741	0.980	[0.733 - 1.311]	0.886
IP-10	1.063	[0.940 - 1.202]	0.312	1.018	[0.878 - 1.181]	0.803
MCP-1	1.088	[0.956 - 1.237]	0.188	0.924	[0.808 - 1.057]	0.232
IL-12	0.452	[0.177 - 1.157]	0.093	0.879	[0.556 - 1.389]	0.560

M6 = month 6, M0 = baseline, CI = confidence intervals

5.8.6 Change in CMV IgG titre levels

Given the fact that CMV IgG titre has been linked with adverse outcomes [278], a pre-specified exploratory outcome was whether valaciclovir treatment for 6 months can lead to a reduction in CMV IgG titres. To determine this, plasma samples were collected from clinical trial participants monthly over the duration of the trial and analysed at the end of the study as outlined in Section 2.6.8. Because initial titre levels varied widely between patients and in order to avoid skewing of the data by occasional patients with high titre values, the data was normalised by expressing it as a percentage at each month relative to the baseline that was given a value of 100%.

The CMV IgG titre percentage levels in control patients remained fairly static for the duration of the clinical trial period (Figure 5.16, Panel B). In contrast, CMV IgG titre percentage levels gradually reduced in the treatment group, although this became more evident from month 5 onwards in keeping with the half-life of IgG (Figure 5.16, Panel A). The CMV IgG percentage titre sharply increased for participant CT005 (red arrow) and CT031 (green arrow) during the treatment period, in contrast to the rest of the treatment group (Figure 5.16, Panel A). Interestingly, CT005 had a CMV reactivation episode at baseline prior to commencing treatment (Table 5.3) suggesting a temporal delay between CMV reactivation and rise in CMV IgG titre. Participant CT031 on the other hand had a 1-week break in treatment during month 2 which was followed by a gradual rise in CMV IgG titre and a further 2-week break in treatment during month 4 that was followed by a CMV reactivation episode of low intensity (Table 5.3) and a further spike in CMV IgG titre in month 7 (Figure 5.16). Similar spikes in CMV IgG titre were noted following CMV reactivation in control patients.

Repeated measures ANOVA revealed a statistically significant change overall in the CMV IgG titre percentage change during the study period in the treatment group ($p=0.040$) but not in the control patients ($p=0.813$). Furthermore, on post-hoc analysis there was a statistically significant negative linear trend in the treated patients (post-test for linear trend, slope -1.305, $p<0.001$) but not in controls (post-test for linear trend, slope 0.218, $p=0.521$) indicating that blocking subclinical CMV reactivation with

valaciclovir led to a statistically significant reduction in CMV IgG titre in the treated patients only.

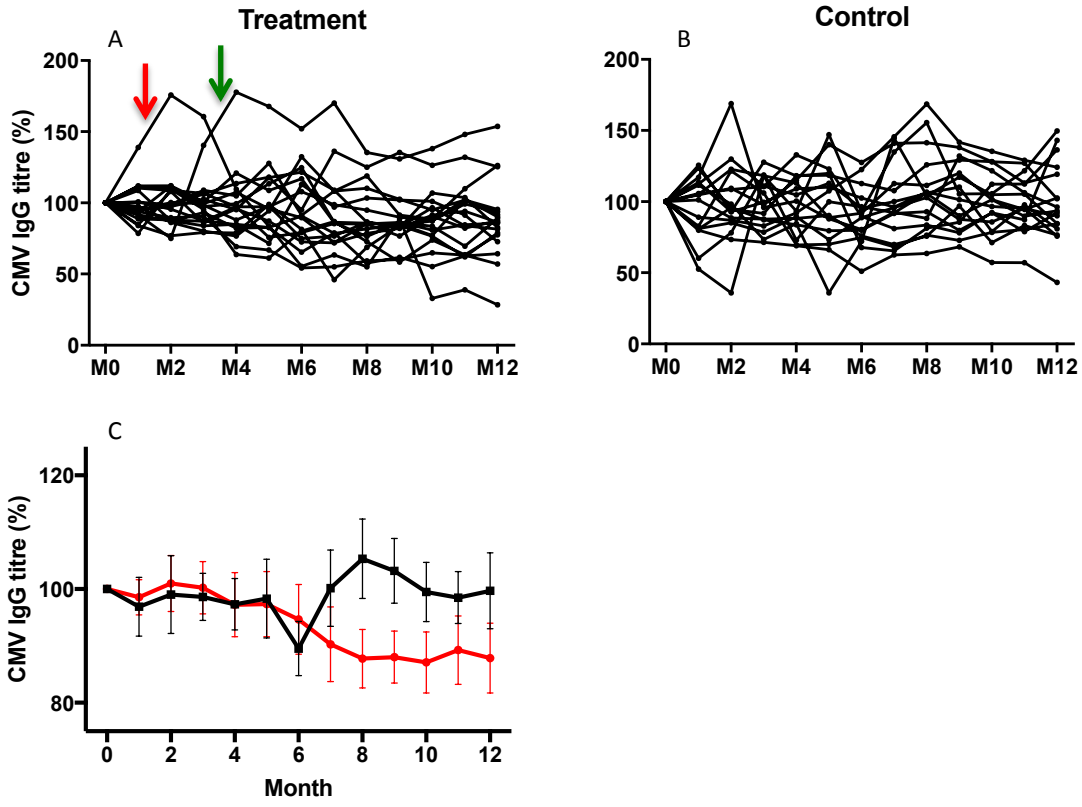

Figure 5.16 Panels A and B show summary data of CMV IgG titre percentage change in treatment (n=19) and control (n=17) patients respectively

The red and green arrows in Panel A refer to participant CT005 and CT031 respectively that displayed a rise in CMV IgG titre following episodes of CMV reactivation. Panel C shows mean IgG titre percentage values in treated (red) and control (black) patients. The IgG titre gradually declined over the course of the study in treated patients but not in controls.

5.8.6.1 Correlation between reduction in CMV IgG titre and CD4+CD28null T-cell parameters

Having observed a reduction in CD4+CD28null T-cell proportion, CD4+CD28null T-cell absolute count and CMV IgG titre in the treated patients, further analysis was carried out to determine whether these changes were appropriately correlated in the same patients. In order to achieve this, linear regression analysis was performed between the month 6 to baseline CD4+CD28null T-cell proportion or absolute count log ratio and the slope of the CMV IgG titre change from baseline to month 12 (Figure 5.17). CMV IgG titre slope was examined across the entire study period due to the observed delay in titre reduction in treated patients.

The change in CD4+CD28null T-cell proportion as well as absolute count was directly related to the change in CMV IgG titre as shown in Figure 5.17. This association was statistically significant in the treated patients but not in controls suggesting that blocking subclinical CMV reactivation with valaciclovir therapy resulted in a reduction in the CD4+CD28null T-cell expansion which was mirrored by a drop in CMV IgG titre adding validity to the results reported earlier in this chapter. Interestingly, although not statistically significant, the change in CD4+CD28null T-cell absolute count appeared to be related to the change in CMV IgG titre also in the control patients (Figure 5.17, Panel D). This probably reflects the influence of subclinical CMV reactivation, or absence of, on the CD4+CD28null T-cell compartment and CMV IgG titre as further discussed below in Section 5.9.

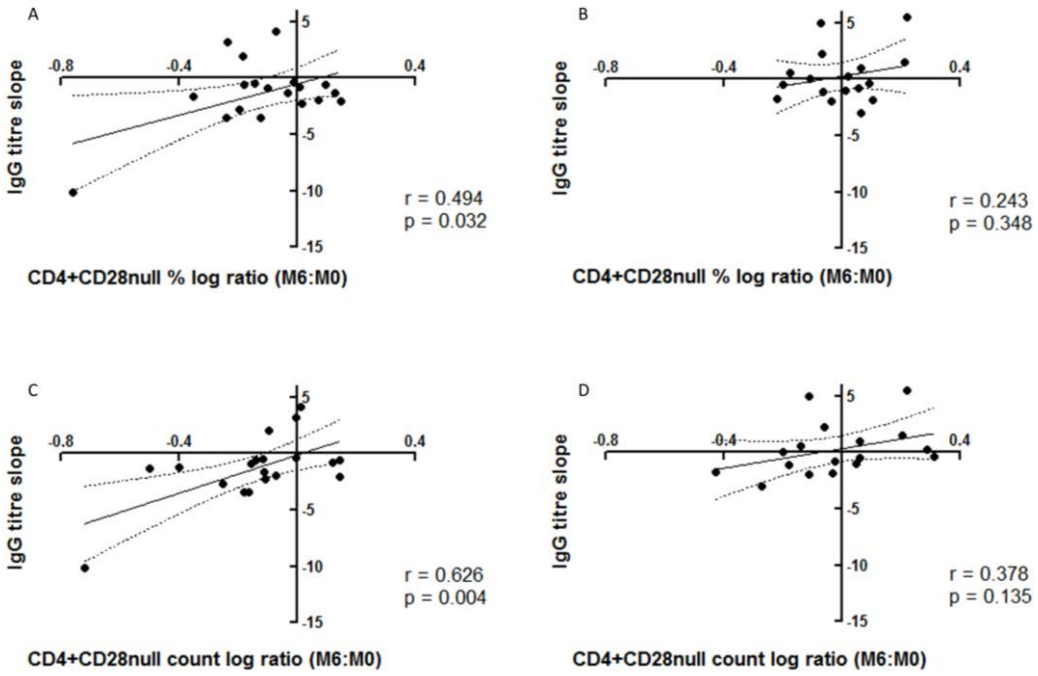

Figure 5.17 Correlation between CMV IgG titre and CD4+CD28null T-cell parameters Linear regression analysis was carried out between the CD4+CD28null T-cell proportion (A, B) or CD4+CD28null T-cell absolute count (C, D) month 6 (M6) to baseline (M0) log ratio and the CMV IgG titre slope change in treated (n=19) (A, C) and control (n=17) (B, D) patients. Regression lines with 95% confidence intervals are shown.

5.8.7 Change in pulse wave velocity across the study period

Given the association between CD4+CD28null T-cell expansion and cardiovascular disease as well as the independent association between CD4+CD28null T-cells and increased arterial stiffness as measured by pulse wave velocity, observed in Chapter 4, the final exploratory outcome of the clinical trial arm aimed to determine whether treatment with valaciclovir for 6 months could lead to an improvement in arterial

stiffness. Arterial stiffness was measured by carotid femoral PWV as detailed in Chapter 2 at baseline, at the end of the 6 month treatment and at the end of the study at 12 months.

Figure 5.18 (Panel A) shows PWV adjusted for a mean arterial pressure (MAP) of 90 mmHg for control and treated patients. There was a statistically significant increase in PWV in the control patients but not the treated patients across the study period suggesting that controlling subclinical CMV reactivation and reducing the size of the CD4+CD28null T-cell expansion might have had a protective effect in the treated patients. Furthermore, the absolute count of CD4+CD28null T-cells at baseline significantly correlated with the change in PWV (adjusted for MAP of 90 mmHg) across the study period in that patients with higher numbers of CD4+CD28null T-cells at baseline exhibited a larger increase in PWV across the study period (Figure 5.18, Panel B).

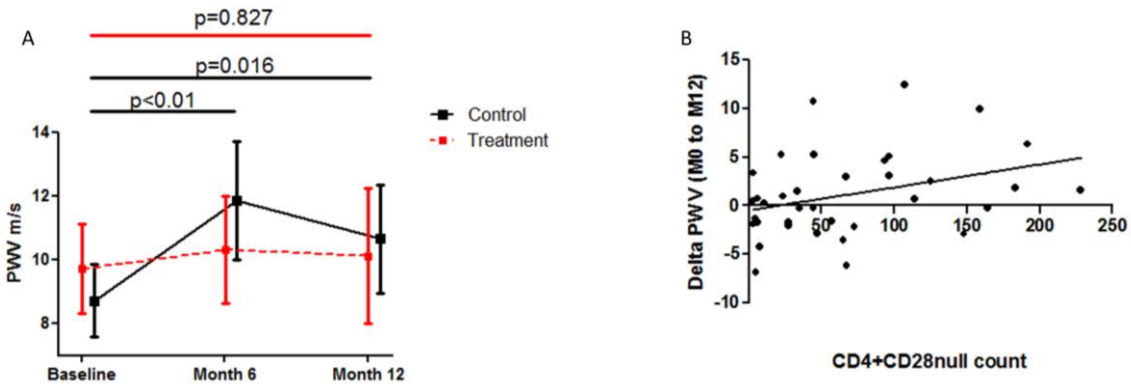

Figure 5.18 Change in pulse wave velocity across the study period

Panel A shows the change in PWV (adjusted for MAP 90 mmHg) across the study period for control (n=19) (black) and treatment (n=19) (red) patients. There was an overall increase in PWV in the control patients (p=0.016) but not the treated ones (p=0.827) with the statistically significant difference in the control patients lying between the baseline and month 6 time points (ANOVA with post-hoc Dunnett's multiple comparison test comparing M12 and M6 to the baseline values). In panel B, the CD4+CD28null absolute cell count at baseline was correlated to the magnitude of the change in PWV (adjusted for MAP 90 mmHg) from baseline to month 12 (r=0.334, p=0.047; Pearson's correlation).

5.9 CMV reactivation and expansion of the CD4+CD28null T-cell compartment in the control patients

In order to further evaluate the impact of subclinical CMV reactivation on the size of the CD4+CD28null T-cell compartment, the change in the size of the CD4+CD28null T-cell expansion across the entire study period was assessed in the control patients (n=19) in relation to CMV reactivation. Patients that reactivated CMV at least once

exhibited an overall increase in CD4+CD28null T-cell percentage (0.9 [IQR -1.1 - 2.0]) compared to those that did not (-1.6 [-8.9 - 0.4]; p=0.038) (Figure 5.19, Panel A). Furthermore, the overall change in CD4+CD28null T-cell percentage over the study period was correlated with the number of reactivation episodes observed (r=0.537, p=0.018) (Figure 5.19, Panel B).

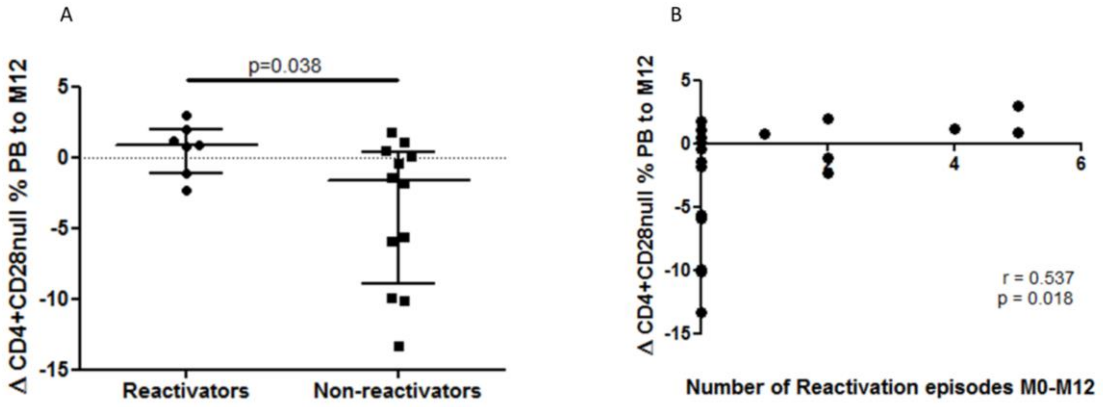

Figure 5.19 CMV reactivation and change in CD4+CD28null T-cells in controls

The change in CD4+CD28null T-cell proportion between the pre-baseline visit (PB) and month 12 (M12) in control patients (n=19) was assessed in relation to CMV reactivation between the baseline and month 12 visits. Patients that reactivated CMV at least once versus those that did not reactivate at all are shown in Panel A (Mann Whitney U test). In Panel B the correlation between the total number of reactivation episodes and the change in the CD4+CD28null T-cell compartment is shown (Spearman's rank test).

Next, the CD4+CD28null T-cell proportion was evaluated monthly across the study period using cryopreserved PBMC samples from 3 control patients that reactivated CMV during follow up. Figure 5.20 shows the CD4+CD28null T-cell percentage plotted against CMV viral copy number and CMV IgG titre. A temporal relationship was observed between episodes of CMV reactivation and changes in the size of the CD4+CD28null T-cell compartment as well as the CMV IgG titre. Measurable CMV reactivation was preceded by an increase in CD4+CD28null T-cell proportion and was followed by a rise in CMV IgG titre in agreement with the results presented so far.

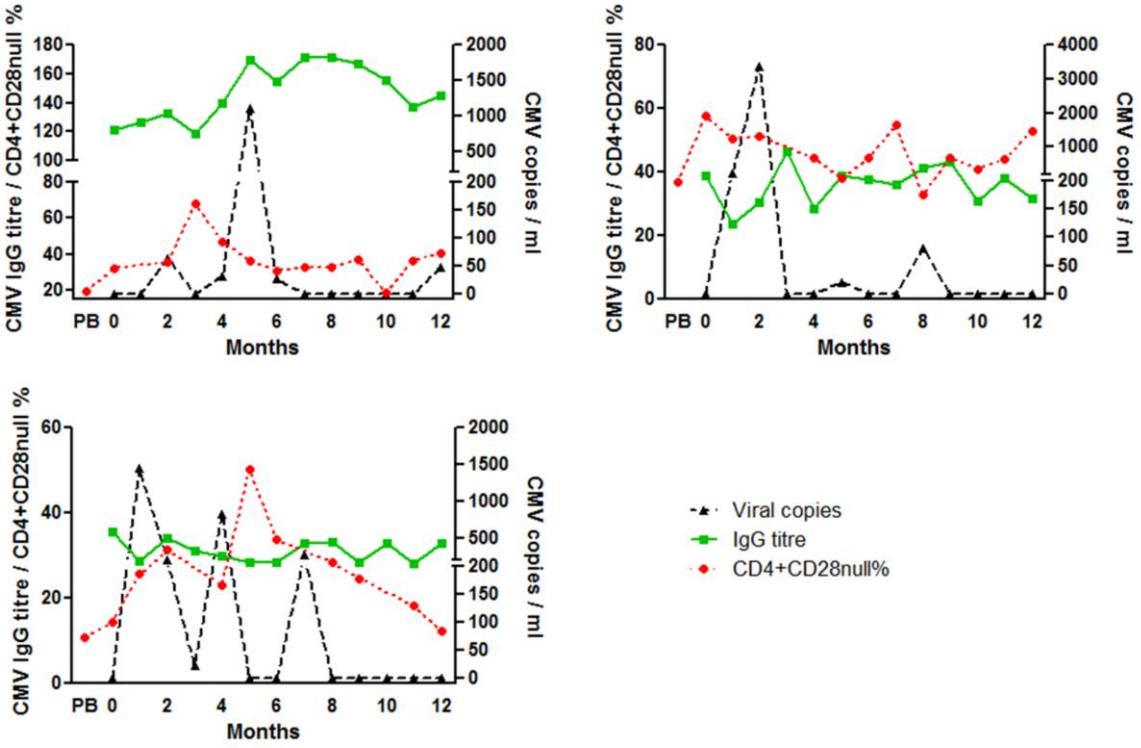

Figure 5.20 CD4+CD28null tracking experiments in control patients with CMV reactivation

The change in CD4+CD28null T-cell proportion was tracked across the study period (pre-baseline (PB) to month 12) in 3 different control patients that reactivated CMV in order to relate the onset of CMV reactivation (right y axes) to changes in CD4+CD28null T-cell proportion and CMV IgG titre (left y axes). An increase in CD4+CD28null T-cell percentage preceded measurable CMV reactivation. CMV reactivation was followed by an increase in CMV IgG titre in some but not all episodes of reactivation.

5.10 CMV reactivation and polyfunctionality

The data discussed in Chapter 3 revealed that CD4+CD28null T-cells, despite being markedly pro-inflammatory, were less polyfunctional in terms of IFN-γ, TNF-α and IL-2 co-expression following overnight stimulation with CMV lysate compared to their CD4+CD28+ counterparts. The effect of relative proportions of polyfunctional cells on the risk of CMV reactivation was therefore evaluated. Patients free from CMV reactivation over the 12 month study period had a higher baseline ratio of CD4+CD28+ to CD4+CD28null CMV specific T-cells (Figure 5.21, Panel A). In addition, this ratio was negatively correlated to the total number of reactivation episodes over the 12 month study period (Figure 5.21, Panel B).

Finally, patients that reactivated CMV at least once had a lower baseline proportion of polyfunctional CD4+CD28+ CMV specific T-cells (Figure 5.21, Panel C), defined as CD4+CD28+IFN-γ+ cells also capable of TNF-α and IL-2 co-expression following overnight stimulation with CMV lysate suggesting that the possession of polyfunctional CD4+CD28+ CMV specific T-cells is important in keeping CMV controlled and avoiding episodes of subclinical CMV reactivation.

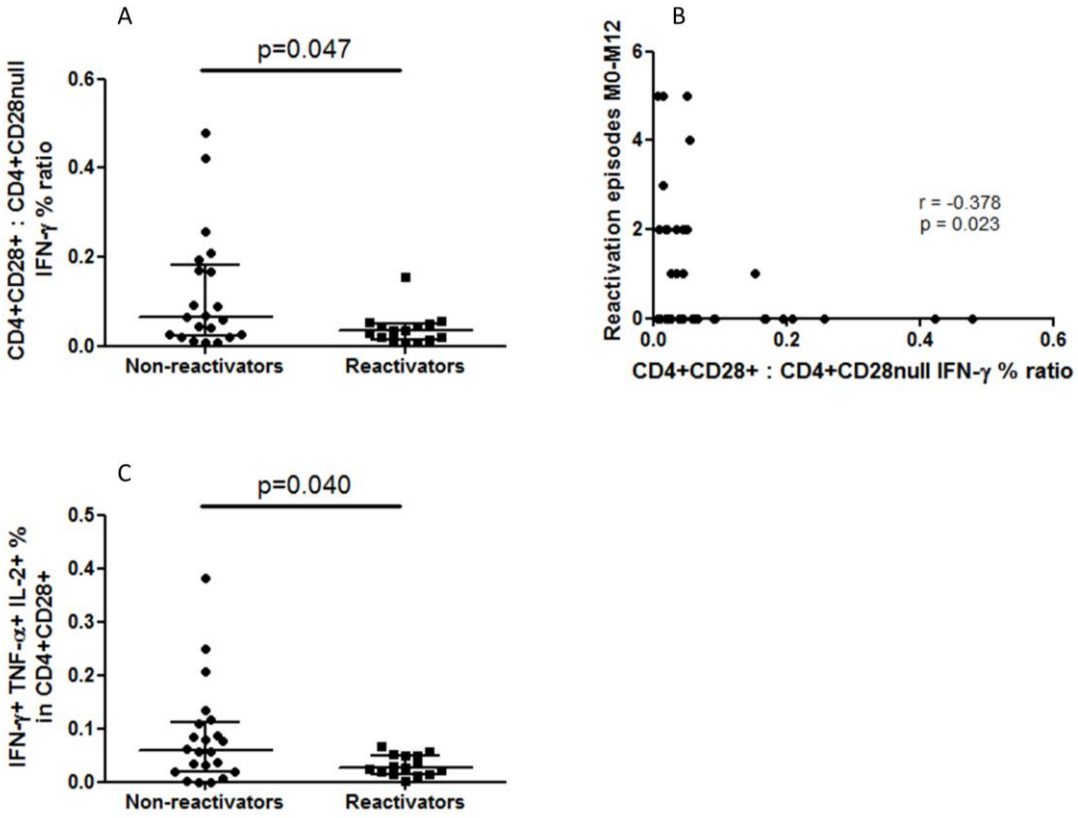

Figure 5.21 Effect of polyfunctionality on risk of CMV reactivation (n=38)

Panel A: Patients that reactivated CMV at least once over the course of the study had a lower ratio of CD4+CD28+:CD4+CD28null CMV specific T-cells at baseline, identified by IFN-γ expression following overnight stimulation with CMV lysate (Mann Whitney U test). Panel B: The ratio of CD4+CD28+:CD4+CD28null CMV specific T-cells at baseline was negatively correlated with the total number of CMV reactivation episodes over the study period (Spearman's rank test). Panel C: Patients that reactivated CMV at least once over the course of the study had a lower proportion of polyfunctional CD4+CD28+ CMV specific T-cells, identified as CD4+CD28+IFN-γ+ T-cells capable of TNF-α and IL-2 co-expression following overnight stimulation with CMV lysate compared to patients that did not reactivate.

5.11 Discussion

There is very little information in the literature on the prevalence and magnitude of potential CMV reactivation during latency in immunosuppressed patients with inflammatory conditions. The data presented here show that significant subclinical CMV reactivation does indeed occur in CMV seropositive AAV patients in disease remission and that 37% of patients will reactivate CMV at least once over a period of 12 months based on reactivation rates in the control patients. All detectable episodes were asymptomatic, of low titre and were mainly seen in patients' urine.

Six months treatment with valaciclovir was successful in blocking CMV reactivation in AAV patients. In turn, blocking CMV reactivation in treated patients led to a consistent reduction in several parameters of CMV-specific cell mediated immunity compared to controls that showed no change in any of these assessments. Specifically, the proportion as well as absolute count of CD4+CD28null T-cells was reduced after 6 months of valaciclovir therapy in treated patients but not in controls. In addition, the absolute count of CD8CD28null T-cells as well as the proportion and absolute count of CD4+CD28null CMV-specific T-cells were reduced in treatment but not in control patients. The fact that blocking CMV reactivation with valaciclovir in treated patients led to a decrease in these cell populations indicates that expansion of such T-cell subsets is driven by episodes of subclinical CMV reactivation.

This is supported by the supplementary analysis in the control patients where subjects that reactivated CMV showed an increase in their CD4+CD28null T-cell percentage. The increase in CD4+CD28null T-cell percentage in control patients was also found to be correlated with the number of reactivation episodes over the study period. Detailed analysis of the change in CD4+CD28null T-cell percentage on a per month basis in a subset of control patients that reactivated CMV showed that a rise in CD4+CD28null T-cell percentage preceded the onset of documented reactivation.

The reduction in CMV-specific CD4+CD28null T-cells capable of producing IFN-γ following overnight stimulation with CMV lysate in treated patients was accompanied by a reduction in IL-2 and IFN-γ plasma levels that was not seen in controls. The plasma marker of endothelial dysfunction P-selectin was also reduced only in treated patients. P-selectin is expressed on inflamed endothelium and is implicated in vascular inflammation where proinflammatory Th1 T-cells bearing its ligand PSGL-1 (P-selectin glycoprotein ligand 1) under control of the transcription factor T-bet are able to bind to it and induce endothelial damage [353, 358]. As such, a reduction in P-selectin and Th1 mediated cytokines seen here may translate to an improvement in vascular inflammation. There was a suggestion from the serial pulse wave velocity measurements undertaken during the trial that arterial stiffness increased in the control patients but remained static in the treated patients during the first 6 months of the study. However, the trial was not designed to detect a difference in pulse wave

velocity between treated and control patients and therefore this remains to be adequately investigated in future studies.

Blocking subclinical CMV reactivation with valaciclovir also led to a sustained reduction in CMV IgG titre levels. There was a temporal delay associated with this, which is in keeping with the findings presented in Figure 5.15 (Panel A), where CMV reactivation preceded a rise in IgG titre that could be detected in peripheral blood. Similarly, the effect of blocking CMV reactivation with valaciclovir treatment on the CMV IgG titre became clear only at month 5 and persisted for several months following treatment cessation likely as a result of the long half-life of IgG [359].

Importantly, the decline in CMV IgG titre in treated patients was mirrored by a decrease in CD4+CD28null T-cell percentage and absolute counts as these changes were correlated when plotted against each other. In addition, changes to CMV IgG titre levels have been shown to correspond to episodes of reactivation [360-362]. Hence, a declining CMV IgG titre in the treated patients likely reflects another marker of evidence of blocked CMV reactivation in addition to the PCR results and the fact that the CMV IgG titre correlated with a drop in CD4+CD28null T-cell indices of similar magnitude adds further validity to the data.

Valaciclovir was well tolerated with mainly gastrointestinal symptoms reported by treated patients in keeping with the drug's known side effect profile. There was an episode of significant valaciclovir toxicity associated with acute kidney injury in one patient. This was picked up at the month 1 safety assessment, however on discussion with the patient the symptoms had started from the 2nd week of taking the drug. Future clinical trials should therefore include a safety assessment also at 2 weeks as significant toxicity, where it occurs, is likely to be encountered within the first few weeks of commencing treatment.

As discussed, there was a consistent effect of valaciclovir treatment leading to a block of CMV reactivation which was associated with a reduction in CD4+CD28null T-cells and other subsets of CMV-specific and associated cell mediated immunity, plasma levels of markers of inflammation and endothelial dysfunction and plasma CMV IgG titres. Importantly, this treatment effect was consistently found across 4 different techniques that were used in the assessment of these outcomes namely, qPCR, flow cytometry, LUMINEX and ELISA. The clinical trial was powered to detect a difference in reactivation rates between treated and control patients. Hence 2-way ANOVA analyses comparing repeated measure assessments between treated and control patients for the secondary and exploratory outcomes were not carried out. However, the fact that a reduction in all secondary and exploratory outcomes of CMV mediated changes to the immune system was persistently seen in the treated patients only and not in the control group is in keeping with validity of these findings. The results of the

trial are very encouraging. However, there is a need to replicate these findings in a larger clinical trial adequately powered to detect statistically significant changes in these parameters with 2-way ANOVA analyses.

There are some learning points from this study that can be applied to a future larger clinical trial. There was a discrepancy noted in the CD4+CD28null T-cell percentage between patients randomised to the treatment versus the control arm although this difference was not statistically significant. A CD4+CD28null cell percentage stratification cut-off of 40% was incorporated into the randomisation algorithm. However, there were only a couple of patients with CD4+CD28null cell percentages greater than 40% so follow up clinical trials should utilise a lower stratification cut-off in order to achieve better balance between treated and control patients.

Furthermore, given the longevity of CD4+CD28null T-cells and the delay in the reduction of CMV IgG titre observed in this trial, it is possible that a longer duration of anti-viral treatment is necessary to attain larger reductions in these pro-inflammatory T-cells rather than the modest reductions observed in this present study. Future trials should therefore employ a duration of treatment of 12 months in order to assess whether this can be achieved and also evaluate clinically meaningful outcomes such as change in arterial stiffness that are likely to require a longer treatment and follow up period to detect significant such changes.

In summary, this proof of concept clinical trial presented here shows that CMV reactivation regularly occurs in AAV CMV seropositive patients in remission and can be safely blocked with valaciclovir treatment. The data shows for the first time that subclinical CMV reactivation drives the expansion of CD4+CD28null T-cells and that this and other associated CMV induced changes to the immune system can be ameliorated with valaciclovir treatment in patients with AAV, in agreement with the study hypothesis.

CHAPTER 6

GENERAL DISCUSSION

Chapter 6 General Discussion

The two leading causes of morbidity and mortality in AAV are infection and cardiovascular disease [93]. Previous research has shown that large expansions of proinflammatory CD4+CD28null T-cells in AAV patients are only present in CMV seropositive individuals and are associated with increased infection, reduced renal function and increased mortality [112]. CMV and CD4+CD28null T-cells have also been described as having a significant bearing on immunosenescence and are associated with reduced immune responses to heterologous antigen [297] and influenza vaccination [300]. In addition CMV seropositivity and CD4+CD28null T-cell expansions have been linked to vascular pathology [287] and an increased risk of cardiovascular disease in patients with inflammatory conditions such as chronic kidney disease (CKD), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [156, 175, 180].

This thesis examined the hypothesis that subclinical CMV reactivation is the driver behind the observed expansion of CD4+CD28null T-cells in inflammatory disease. Part of the work carried out aimed to explore the mechanisms via which the harmful effects of this cell subset are mediated in patients with AAV. In addition, a proof of concept open label randomised controlled clinical trial of 6 months oral valaciclovir or no additional therapy in CMV seropositive AAV patients in remission was designed and implemented during the course of this PhD. The trial tested the hypothesis of whether

blocking subclinical CMV reactivation with valaciclovir can halt or reduce the expansion of CD4+CD28null T-cells and ameliorate associated CMV induced immune dysregulation. Further work aimed to explore the mechanisms involved in the control of subclinical CMV reactivation.

6.1 Subclinical CMV reactivation drives the expansion of CD4+CD28null T-cells

The first of its kind proof of concept clinical trial carried out during the course of this work has shown that firstly, valaciclovir blocks subclinical CMV reactivation in CMV seropositive AAV patients and secondly, that this in turn leads to a reduction in the proportion of CD4+CD28null T-cells in treated patients only. Other favourable changes such as reduction in inflammatory and endothelial dysfunction markers and reduction in CMV IgG titres were also seen in the treated patients but not in controls indicating that blocking CMV reactivation with valaciclovir ameliorates associated CMV adverse modulation of the immune system in AAV. Finally, valaciclovir therapy was safe and well tolerated.

As discussed, CMV is implicated in the immunosenescence that is increasingly been recognised with advancing age but may also play a part in immune dysregulation in the setting of inflammatory disease leading to weakened immune responses. One possible mechanism in which this may occur is via 'memory inflation' whereby expansion of

proinflammatory subsets such as CD4+CD28null T-cells is mirrored by a shrinkage of the naïve CD4 T-cell compartment associated with a reduced ability to mount effective responses to new antigens.

Work carried out during this thesis has shown that the size of the CD4+CD28null T-cell compartment in AAV patients is reciprocally related to the CD4:CD8 ratio suggesting that limiting CD4+CD28null T-cell expansion by blocking subclinical CMV reactivation may also have favourable outcomes in terms of improving the 'immune risk profile' in patients with AAV. Indeed, valaciclovir treatment in the clinical trial was associated with an improvement in the CD4:CD8 ratio in treated patients whereas no change was seen in control patients, although these findings need to be confirmed in a larger study.

6.2 Phenotype of CD4+CD28null T-cells in AAV and mechanisms of vascular damage

Several reports exist on the type of surface and intracellular markers possessed by CD4+CD28null T-cells in different inflammatory conditions and in healthy individuals. However a comprehensive analysis of the phenotype of CD4+CD28null T-cells in AAV has not been previously undertaken. The studies presented here have shown CD4+CD28null T-cells in AAV to be CMV responsive Th1 cells with a proinflammatory potential expressing mainly IFN-γ and TNF-α. Further analysis of their cell surface

chemokine receptor pattern showed for the first time that such cells in AAV co-express CXCR3, CX3CR1, CD49d and CD11b suggesting that they are able to traffic to and bind activated endothelial cells. Surface expression of NKG2D and possession of intracellular perforin and granzyme B that have been shown to enable endothelial cell lysis were also seen. Finally, the size of the expansion of CD4+CD28null T-cells in CMV seropositive AAV patients was independently associated with increased arterial stiffness as measured by carotid to femoral pulse wave velocity suggesting involvement of this cell subset in the pathophysiology of atherosclerosis in CMV seropositive patients.

Other researchers have previously shown that during in vitro co-culture experiments, peripheral blood mononuclear cells (PBMC) from CMV seropositive individuals with a high proportion of CMV specific CD4 T-cells induce higher levels of upregulation of fractalkine, VCAM-1 and ICAM-1 (the ligands for CX3CR1, CD49d and CD11b identified on CD4+CD28null T-cells in this thesis) on endothelial cell monolayers and exhibit higher levels of PBMC adherence and endothelial damage compared to PBMC from individuals with a low proportion of CMV specific CD4 T-cells [363]. Endothelial cell activation was dependent on IFN-γ and TNF-α production by CMV specific cells and in other experiments the ligand for CXCR3, IP10, was also shown to be upregulated on activated endothelium and be critical for the adherence of CMV effector CD4 T-cells onto endothelial cell monolayers [287].

Taking this together with the data from this thesis it is proposed that in AAV patients subclinical CMV reactivation drives the expansion of proinflammatory CMV specific CD4+CD28null T-cells that are targeted to endothelial cells through co-expression of unique chemokine receptors. Through production of Th1 cytokines these cells induce endothelial activation and via release of cytotoxic perforin and granzyme B participate in endothelial damage exacerbating atherosclerosis and arteriosclerosis and leading to an increased incidence of cardiovascular disease.

6.3 Control of subclinical CMV reactivation requires polyfunctional CD4 responses

The mechanisms that control subclinical CMV reactivation remain unclear. Given the link between subclinical CMV reactivation and CD4+CD28null T-cell expansion demonstrated by this thesis, it is important to consider how viral control is maintained in this situation. Work undertaken here has identified TIM-3 as a marker of exhausted CD4+CD28null T-cells with reduced functionality. Furthermore, CMV specific CD4+CD28+ cells were identified as having increased functionality compared to CD4+CD28null T-cells.

Analysis of the clinical trial CMV reactivation results revealed that patients reactivating CMV at least once during the course of the study had a lower ratio of CMV specific CD4+CD28+ to CMV specific CD4+CD28null T-cells at baseline and that the number of

viral reactivation episodes was also inversely correlated with this ratio indicating for the first time the importance of polyfunctional CD4+CD28+ CMV specific T-cells in the control of subclinical CMV reactivation. Furthermore, patients that reactivated CMV at least once during the study also had a lower percentage of polyfunctional CD4+CD28+ CMV specific T-cells at baseline further highlighting the importance of multiple-cytokine producing T-cells in the control of subclinical reactivation. It will be interesting to see in subsequent studies whether controlling subclinical CMV reactivation changes the pattern of cytokine production in CMV specific T-cells in a favourable way or not.

6.4 Conclusion

In conclusion, the studies undertaken during this PhD have shown for the first time that subclinical CMV reactivation drives the expansion of CD4+CD28null T-cells in a patient group with an inflammatory disease. CD4+CD28null T-cells were shown to be endothelial homing cytotoxic T-cells and were independently associated with increased arterial stiffness, a well-established marker of increased cardiovascular risk. It is proposed here that CD4+CD28null T-cells through expression of chemokine receptors bind to respective ligands on activated endothelial cells leading to vascular damage which ultimately culminates to the observed increased risk of cardiovascular disease seen in association with CMV and this proinflammatory subset. Finally, blocking subclinical CMV reactivation with valaciclovir in AAV patients led to a reduction in CD4+CD28null T-cells. This opens exciting potential therapeutic avenues for patients with AAV but also other inflammatory conditions such as chronic kidney disease and rheumatoid arthritis where such CMV driven changes have been associated with adverse outcomes including a higher risk of cardiovascular disease.

6.5 Future work

Chronic kidney disease (CKD) is a global public health concern with an estimated prevalence of over 10% of the population in Western Europe and the United States [364]. People with CKD are much more likely to die from cardiovascular disease (CVD) rather than progress to end-stage renal failure and the risk of CVD in CKD increases inexorably with declining renal function. Furthermore this increased risk cannot be fully attributed to conventional atherosclerotic risk factors and it is likely that increased arterial stiffness is one of the major contributors to this adverse outcome [348].

Previous work has shown that CMV seropositive patients CKD have increased arterial stiffness [285]. In addition, CD4+CD28null T-cell expansions in CKD correlate with measures of early atherosclerotic damage and CMV seropositivity with prevalence of atherosclerotic disease [173, 180, 181]. Given the findings of this thesis we have hypothesised that subclinical CMV reactivation drives the expansion of cytotoxic CD4+CD28null T-cells in patients with CKD leading to increased arterial stiffness, a key contributor of increased CVD in CKD. As such subclinical CMV reactivation and the expansion of CD4+CD28null T-cells may represent a potentially modifiable factor in the treatment of the increased cardiovascular risk in CKD. We are currently in the process of submitting a grant application to carry out an interventional study in patients with CKD of 12 months treatment with valaciclovir in order to determine whether blocking

subclinical CMV reactivation and ameliorating the CD4+CD28null T-cell expansion in this patient group can reduce arterial stiffness.

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Appendix 1 Publications and abstracts arising from this thesis at the time of submission

Publications

1. Chanouzas, D., Dyall L., Nightingale, P., Ferro, C., Moss, P., Morgan, M.D., Harper, L. *Valaciclovir to prevent Cytomegalovirus mediated adverse modulation of the immune system in ANCA-associated vasculitis (CANVAS): study protocol for a randomised controlled trial.* Trials, 2016. **17**(1): p. 338.

Abstracts

1. Chanouzas, D., Sagmeister, M., Dyall, L., Nightingale, P., Ferro, C., Moss, P., Morgan, M.D., Harper, L. *Valaciclovir to prevent cytomegalovirus mediated adverse modulation of the immune system in ANCA associated vasculitis (CANVAS): results from a randomised controlled trial.* Accepted for poster presentation at American Society of Nephrology Congress, Nov 2016, Chicago. USA.

2. Chanouzas, D., Dyall, L., Ferro, C., Moss, P., Morgan, M.D., Harper, L. *CD4+CD28- T-cells are CMV specific cytotoxic endothelial targeting cells and are independently associated with increased arterial stiffness in renal disease.* Accepted for poster presentation at American Society of Nephrology Congress, Nov 2016, Chicago, USA.

3. Chanouzas, D., Dyall L., Dale, J., Moss, P., Morgan, M.D., Harper, L. *CD4+CD28- T-cell expansions in ANCA-associated vasculitis and association with arterial stiffnes: baseline data from a randomised controlled trial.* Lancet, 2015. **385** Suppl 1:S30.

   Presented as poster presentation at:

   Academy of Medical Sciences Spring Conference, Feb 2015, London

   Abstract 3 was also presented as oral presentation at:

   5th International Workshop on CMV and Immunosenescence, Nov 2014, Amsterdam, Holland

Appendix 2 Study Protocol

University of Birmingham

University Hospital Birmingham NHS Foundation Trust

CANVAS

CMV Modulation of the Immune System in **AN**CA-Associated **VAS**culitis

Study Protocol
Version 3.0
12th August 2015

EudraCT Number: 2012-001970-28
ClinicalTrials.gov Identifier: NCT01633476

Supported by wellcome trust

VASCULITIS UK

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REC Reference Number

12/YH/0377

CTA Reference

2012-001970-28

Protocol Code

CMV-001

This protocol describes the CANVAS study and provides information about procedures for entering participants: it should not be used as a guide for the treatment of other individuals. Every care has been taken in the drafting of this protocol, but corrections or amendments may be necessary.

This study will adhere to Good Clinical Practice and will be conducted in compliance with this protocol, the Data Protection Act, and other regulatory requirements, as appropriate.

TABLE OF CONTENTS

1	**SUMMARY**		10
	1.1	Title	10
	1.2	Aims	10
	1.3	Outcome Measures	10
	1.4	Population	10
	1.5	Key Eligibility Criteria	11
	1.6	Treatment	11
	1.7	Duration	11
	1.8	Study Diagram	12
2	**INTRODUCTION**		
	2.1	Background	13
	2.2	Hypothesis	13
3	**AIMS**		15
4	**DESIGN**		16
	4.1	Plan of Investigation	16
	4.2	Schedule of Assessments	18
	4.3	Outcome Measures	22
	4.4	Study Timeline	22
5	**PARTICIPANT ENTRY**		23
	5.1	Recruitment	23
	5.2	Pre-randomisation evaluations	23
	5.3	Sample size	23
	5.4	Inclusion Criteria	24
	5.5	Exclusion Criteria	24
	5.6	Withdrawal Criteria	24
6	**RANDOMISATION PROCEDURE**		26
7	**TREATMENT**		27
	7.1	Treatment Arms	27
	7.2	Dose Modifications for Toxicity	27
	7.3	Interactions with Other Drugs	28
	7.4	Monitoring of Renal Function	28
	7.5	Dispensing	29
8	**TRIAL MANAGEMENT**		30
	8.1	Roles and Responsibilities	30

	8.2	Trial Steering Committee	30
	8.3	Trial Closure	30
9	**PHARMACOVIGILANCE**		31
	9.1	Definitions	31
	9.2	Causality	32
	9.3	Reporting Procedures	33
10	**ASSESSMENT AND FOLLOW-UP**		34
	10.1	Loss to follow-up	34
11	**STATISTICS AND DATA ANALYSIS**		35
	11.1	Statistical Plan	35
	11.2	Data Handling, Record Keeping and Retention	35
	11.3	Data Access	36
	11.4	Case Report Forms	36
12	**MONITORING**		37
	12.1	Risk Assessment	37
13	**REGULATORY ISSUES**		38
	13.1	Clinical Trial Authorization	38
	13.2	Ethics Approval	38
	13.3	Patient Consent	38
	13.4	Confidentiality	38
	13.5	Indemnity	39
	13.6	Sponsor	39
	13.7	Funding	39
	13.8	Audits	39
14	**FINANCIAL ARRANGMENTS**		40
	14.1	Participant Payments	40
15	**REFERENCES**		41
16	**APPENDICES**		43
	A.	Chief Investigator Signature	43
	B.	Signed Letter of Sponsorship Approval	44
	C.	NIC Common Terminology Criteria for Adverse Events	45
	D.	Declaration of Helsinki	46
	E.	Pharmacovigilance Algorithm	51
	F.	Serious Adverse Events Reporting Form	52

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1 TRIAL SUMMARY

1.1 Acronym

CANVAS – CMV modulation of the immune system in ANCA-associated VAScultis

Title

Does CMV reactivation cause functional impairment of CMV specific CD4+ T-cells? The potential for valaciclovir to prevent CMV-mediated adverse modulation of the immune system in patients with ANCA-associated vasculitis

1.2 Aims

Aim 1 (Observational Study)

- To investigate the association between CMV reactivation and functional impairment of CMV-specific CD4+ T-cells

Aim 2 (Clinical Trial)

- To determine whether valaciclovir inhibition of CMV reactivation improves CMV-specific CD4+ T-cell function

1.3 Outcome Measures (for Aim 2)

Primary Outcome

- Proportion of patients with CMV reactivation, as assessed by measurable viral load on quantitative blood or urine CMV PCR.

Secondary Outcomes

- Safety as defined by side effects sufficient to stop treatment with trial drugs or serious adverse events and suspected unexpected serious adverse reactions (SUSARs).
- Change in immune phenotype of the CD4+ CMV specific T cell population at 6 months
- Change in markers of inflammation including serum concentrations of pro and anti-inflammatory cytokines (TNF, IFN, IL-2, IL-6, IL-10, IL-17), markers of systemic inflammation (highly sensitive CRP, ESR)

Tertiary Outcome

- Persistence of valaciclovir effect on immune phenotype of CD4+ T cells at 6 months post treatment

1.4 Population

Aim 1

- 30 CMV seropositive ANCA-associated vasculitis patients (Patient Cohort - Aim 1) and 30 healthy controls (Control Cohort - Aim 1)
- Patient Cohort in Aim 1 will be comprised of the 25 patients randomised to the Control Group in Aim 2 plus 35 additional patients that will be recruited after randomisation has been completed for the entire 50 patients of the clinical trial

- In addition, a follow-up, 'long-term' cohort comprised of 20 patients that took part in a study 9 years ago will be recruited to examine the stability of CMV modulation of the immune system

Aim 2 (Clinical Trial)

- 50 CMV seropositive ANCA-associated vasculitis patients will be randomised to two groups of 25 (Treatment Group will receive valaciclovir whilst Control Group will receive no additional treatment)

1.5 Key Eligibility Criteria (excluding follow-up cohort in Aim 1)

- Age 18 years or over
- CMV seropositive
- Documented diagnosis of ANCA-associated vasculitis (excluding healthy volunteer group)
- In stable remission (no documented clinical disease activity) for at least 6 months prior to entry (excluding healthy volunteer group)

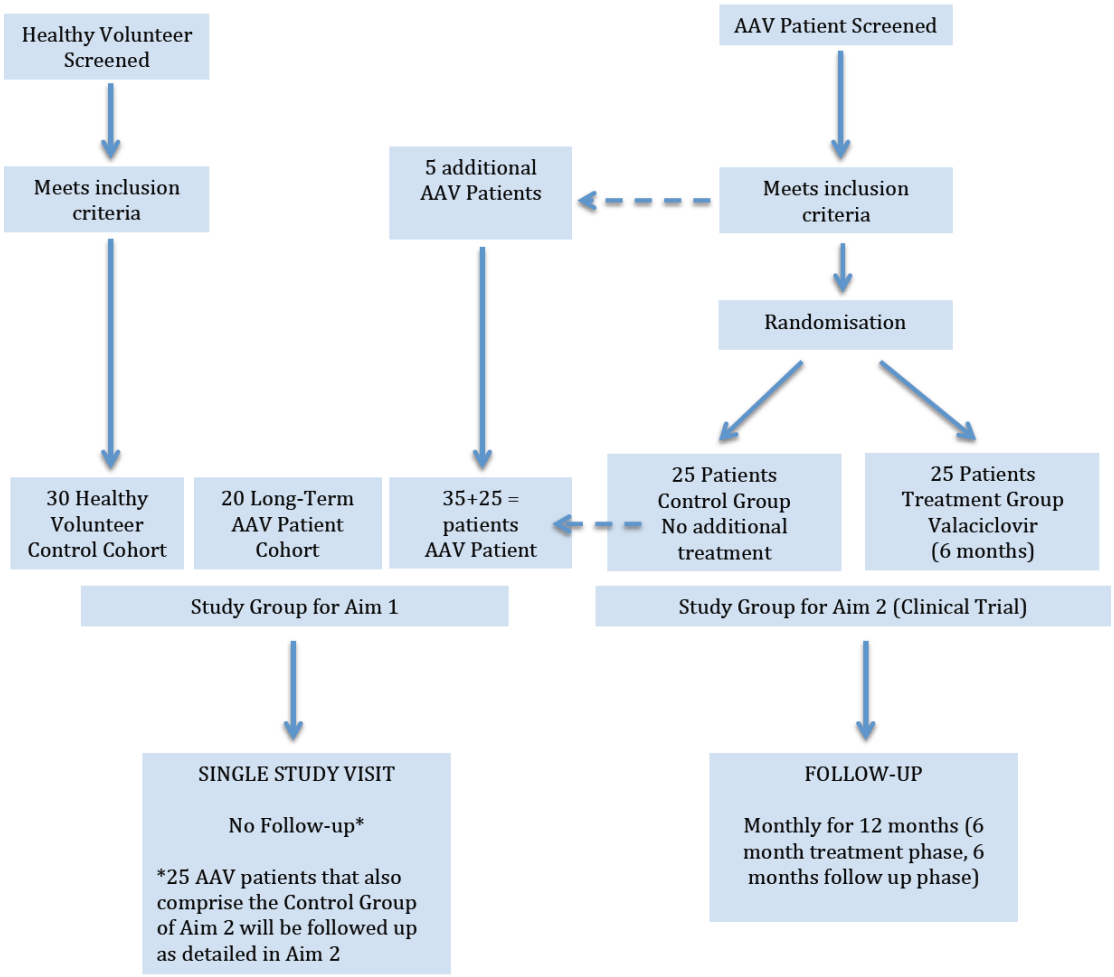
1.6 Treatment

For the clinical trial part of this study (Aim 2) 50 patients with ANCA-associated vasculitis will be recruited and randomised into two groups receiving either valaciclovir at 2000 mg q.d.s or no additional treatment.

1.7 Duration

Patients recruited in Aim 1 of the study as well as the long-term cohort of Aim 1 will attend for a single study visit and will not require follow up. Healthy volunteers recruited in Aim 1 of the study will attend for an initial screening visit followed by a single study visit and will also not require follow up. Patients recruited in the clinical trial part of the study (Aim 2) will be on treatment for 6 months and followed up for a further 6 months.

1.8 Study Diagram



2 INTRODUCTION

2.1 Background

Antineutrophil cytoplasmic antibody (ANCA)-Associated Vasculitides (AAV) are chronic inflammatory diseases with high mortality; infection being the commonest cause of death[1,2]. An increased percentage of CD4+CD28- T-cells with an effector memory phenotype is reported in AAV [3]. We have demonstrated that this phenotype is driven by CMV infection [4]. In our cohort, an expanded CD4+CD28- T-cell population was associated with increased risk of infection and mortality. In transplant recipients the presence of CD4+CD28- T-cell expansion is associated with reduced response to antigenic challenge [5] and in elderly donors this pattern is also associated with frailty and increased mortality [6]. As such considerable evidence suggests that CD4+CD28- T-cell expansion is associated with systemic impairment of immune function.

CMV reactivation is believed to occur on a relatively frequent basis *in vivo* [7-9] and is thought to result in gradual accumulation of CD4+CD28-CMV-specific effector T-cells and reduction in CD4+ naive cells, a phenomenon known as 'memory inflation' [10]. The cytokine production of CMV-specific cells may also be modulated over time correlating with loss of function. Following repeated antigen stimulation with viruses, such as HIV, cytokine production is progressively reduced in a hierarchical manner. IL-2 production and cellular proliferative capacity is lost first, followed by TNF-α and finally IFN-γ [11]. In a study of elderly CMV+ve patients up to 50% of CMV-specific CD4+ T-cells were unable to produce IL-2 and only capable of IFN-γ [12]. Loss of IL-2 production by CMV specific CD4+T-cells is associated with increased risk of CMV end-organ disease in seropositive individuals [13]. Associated with cytokine modulation is an increase in the expression of inhibitory receptors, CTLA-4, PD-1 and TIM-3 leading to attenuation of virus specific T-cell function [11]. In HIV seropositive patients [14] and in transplant patients with CMV viraemia [15] inhibitory receptors are expressed on CD4+ CMV-specific cells, particularly CD4+CD28- T-cells [14], although to a lesser extent than found on CD4+ HIV-specific T-cells [14].

The cytokine production and inhibitory receptor profile of CD4+CD28- T-cells in patients with expansions of this subset have not yet been addressed. The relationship between these features and control of viral replication *in vivo* is also unknown.

Prophylactic valaciclovir treatment can suppress CMV replication [16] and treatment of viraemia reduces PD-1 expression and increases IL-2 production in renal transplant recipients [15]. As such it is likely that valaciclovir treatment could have therapeutic potential in our group of patients.

2.2 Hypothesis

Repeated episodes of CMV reactivation in AAV patients drive the expansion and functional impairment of CD4+CD28-CMV-specific T-cells, with increased susceptibility to infection. Inhibition of CMV replication with valaciclovir will block

further stimulation of CMV specific CD4+ T-cells and increase the functional capacity of the immune system.

3 AIMS

The study has two broad aims.

Aim 1 (Observational Study)
To investigate the association between CMV reactivation and functional impairment of CMV-specific CD4+ T-cells

Aim 2 (Clinical Trial)
To determine whether valaciclovir inhibition of CMV reactivation improves CMV-specific CD4+ T-cell function

4 DESIGN

4.1 Plan of Investigation

One hundred and five patients will be recruited to the study, 25 randomised to each arm of the trial (aim 2); the Control Group plus 35 additional patients will constitute the Patient Cohort in aim 1. The 35 additional patients for Aim 1 will be recruited after the 50 trial patients have been randomised. In addition 20 long-term cohort patients will be recruited as described below.

Aim 1

The association between CMV reactivation and functional impairment of CMV-specific CD4+ T-cells

Following informed consent blood for T-cell analyses will be taken from CMV seropositive AAV patients in remission (n=60) and healthy controls (n=30). We currently follow 200 patients in our vasculitis clinic, of which approximately 70% are CMV+ve. The use of controls will allow us to interpret the influence of immunosuppressive therapy. Preliminary data from this study has shown that detectable CMV reactivation amongst AAV patients is less common than anticipated. Therefore individuals in Aim 1 will attend for a single study visit with the exception of the 25 AAV patients that also comprise the Control Group for Aim 2 (Clinical Trial). In these 25 AAV patients CMV reactivation will be determined by blood and urine PCR monthly for 6 months as detailed in Aim 2 (Amendment 005).

The number and percentage of peripheral blood CD4+CD28- T-cells, and their phenotype will be determined by multi-parameter flow cytometry using antibodies to CD3, CD4, CD27, CD28, CD45RA, CD45RO and CCR7.

The functional capacity of CMV specific CD4+ T-cells will be examined by intracellular cytokine flow cytometry following stimulation with CMV lysate. Change in CD154 expression following CMV lysate stimulation will identify CMV specific cells[17]. The number of CMV specific cells producing IFN-γ, TNF-α, IL-2, IL-5 and IL-10 will determine the proportion of cells with multi-functional cytokine production capacity. The central memory, effector memory and highly differentiated memory phenotype of CD154+ antigen-specific cells as well as their expression of the inhibitory receptors PD-1, CTLA-4 and TIM-3 will be examined.

The association between expansion of the CD4+CD28- T-cells and time to CMV viraemia will be tested using Cox regression survival analysis. Further associations between the functional capacity and phenotypes of the CMV-specific cells and CMV reactivation will be examined.

Use of MHC Class II tetramers to identify CMV specific CD4+T-cells

Our initial data suggest many of the CD4+CD28- CMV-specific T-cells may have an exhausted phenotype characterised by loss of all cytokine production, and may therefore not be detectable by the cytokine detection assay. The use of new HLA Class II tetramer reagents containing peptides which have shown tetramer binding in control subjects (Pachnio, Moss unpublished observations) will allow us to detect the number and phenotype of CD4+ T-cells specific for immunodominant peptides in patients and controls irrespective of their ability to produce cytokine. This is novel work that has not previously been performed. Magnetic bead enrichment of tetramer positive CD4+ T-cells will increase the sensitivity of detection[18]. The phenotype of tetramer-binding cells will then be interrogated in relation to inhibitory biochemical pathways that have been shown to be modulated by chronic stimulation such as PD-1, CTLA-4, LAG-3 and TIM-3 expression. We will also investigate the relationship of transcription factors, T-Bet and BLIMP-1, which have been shown to be associated with CD8+ T-cell inhibitory receptor expression[19]. Cytokine analysis of these cells is not technically possible[20].

CD4+T-cell response to heterologous antigen

We have shown an increased risk of infection associated with CD4+CD28- T-cell expansion[21]. An important question that arises from this data is whether CD4+CD28- expansion is associated with impaired CD4+ T-cell immunity to other viruses. Our group has shown that the CD8+ immune response to CMV can impair the response to other heterologous viruses[21]. We will investigate whether the expansion of CD4+CD28- T-cells impairs CD4+ T-cell response to other viruses using previously described methods[22].

Long-term stability of the CD4+CD28- expansion

We have previously shown that in a cohort of AAV patients, expansion of CD4+CD28- T-cells is driven by CMV [4]. We will reassess the state of the expansion of CD4+CD28- T-cells in 20 patients from a patient cohort that are still under clinical follow up. This will enable us to ascertain whether this expansion can change over time and if so, what the nature of this change is from a quantitative and a qualitative point of view. This long-term cohort will include CMV positive as well as CMV negative individuals.

Influence of CMV on cardiovascular risk

Recently cytomegalovirus seropositivity has been associated with increased arterial stiffness in patients with chronic kidney disease[23], which in turn is linked to cardiovascular risk. We will therefore measure participants' pulse wave velocity, pulse wave analysis and blood pressure using the Vicorder device with added cardiovascular software at baseline, 6 months and 12 months (participants enrolled in clinical trial – Aim 2). This will enable us to determine whether valaciclovir treatment has an effect on such cardiovascular indices. This will be an exploratory outcome and as such will not form part of the main outcomes of the clinical trial described below. In addition we will measure pulse wave velocity, pulse wave analysis and blood pressure using

the Vicorder device also for participants in the control and healthy volunteer as well as long-term cohort groups (single study visit).

Schedule of Assessments (Aim 1)
Healthy volunteers will attend for an initial screening visit. At this visit a single 6ml blood sample will be drawn to determine whether the individual is CMV seropositive. The sample will be analysed at University of Birmingham Laboratories.

CMV seropositive eligible healthy volunteers as well as AAV patients within Aim 1 will attend for a single study visit. The schedule of assessments during this visit is outlined below:

- 50ml blood sample
  - 4ml EDTA tube → WTCRF Lab → Process and store plasma
  - 46ml → SCS Clinical Trials Lab
    - Assessment of CD4+CD28- %
    - Immune assessments
    - Process and store cells
- 50ml urine sample (processed as in Aim 2)
- Pulse wave velocity measurements

The 25 AAV patients that also comprise the Control Group in Aim 2 will also attend for the remainder of the study visits as outlined in Aim 2.

Aim 2 (Clinical Trial)

Does valaciclovir inhibition of CMV reactivation improve CMV-specific CD4 T-cell function?

The clinical trial part of the study will investigate whether short-term treatment with valaciclovir leads to a reduction in CMV reactivation and improved function of CMV-specific CD4+ T-cells. This will be an open label prospective randomised pilot study of valaciclovir or no adjuvant treatment in 50 CMV seropositive patients with AAV who are in remission on a maximum of two immunosuppressant agents. No placebo will be used as this is a pilot project looking at proof of concept and we are using hard end-points for primary and secondary outcomes, which are unlikely to be affected by patient or physician knowledge of treatment. Patients will be recruited from the Vasculitis Clinic at UHB NHS Foundation Trust.

Patients randomised to treatment will be prescribed valaciclovir 8g per day (reduced dose appropriate for renal function) for 6 months with a further 6 month follow up period.

4.2 Schedule of Assessments (Aim 2)

Evaluation of CMV reaction by DNA PCR of urine and blood will be done monthly. Evaluation of the tolerability of drug and adverse events will occur

monthly. Immune assessments as above will be performed at entry, 6 months and 12 months.

Following informed consent patients will undergo an initial pre-baseline visit. During this visit a 5ml blood sample will be drawn. The sample will be analysed at the School of Cancer Sciences Clinical Trials Laboratory (SCS Clinical Trials Lab) to determine the percentage of CD4+CD28- T-cells in the blood. Following this result the patient will be randomized according to the procedure described in Section 6.

At the baseline, 6-month and 12-month visits, a total of 50ml of blood sample will be drawn in addition to a 50ml sample of urine. The blood samples will be analysed and processed by the University Hospital Birmingham Virology Laboratory (UHB Virology Lab) to determine CMV reactivation (primary outcome), the WTCRF Laboratory to isolate and store plasma (later to be used for secondary outcome assays) and the SCS Clinical Trials Lab to determine the percentage of CD4+CD28- T-cells in the blood (secondary outcome), carry out immune assessments as described in Aim 1 (research outcomes) and process and store cells (research outcomes) as some assessments will be batched together near the end of the study. The urine sample will be split in 2 aliquots with 1 part being processed by UHB Virology Lab to determine CMV reactivation (primary outcome) and the other part processed and stored by the WTCRF Laboratory (research outcomes).

The pulse wave velocity measurements as described in section 4.1 will be performed during the baseline, 6-month and 12-month visits (research outcome).

During the remainder of the monthly visits, a 10ml blood sample will be drawn in addition to a 50ml sample of urine that will be processed and analysed by UHB Virology Lab to determine CMV reactivation and the WTCRF Laboratory to process and store. A detailed framework of sample collection procedures and instructions can be found in the Sample Collection Work Instruction that is filed in the Trial Master File (see also Sample Flow Diagram below).

In addition, safety blood tests as described in section 7.4 will be performed for the duration of IMP treatment for those patients randomized to receive the drug (see Schedule of Assessments Diagram below).

Appointments will be scheduled on a monthly basis with a buffer allowance of 10 days either side of the estimated due date of the monthly appointment in order to allow for patient convenience and flexibility. The overall treatment period (or control period for control arm patients) of 6 months will however also be subject to the same 10-day rule in order to avoid excessive movement of the length of treatment in either way. This is detailed in the Appointment Scheduling SOP that is filed in the Trial Master File.

Schedule of Assessments Diagram (Aim 2)

Patient fulfils eligibility criteria for clinical trial

Patient consented to participate in clinical trial

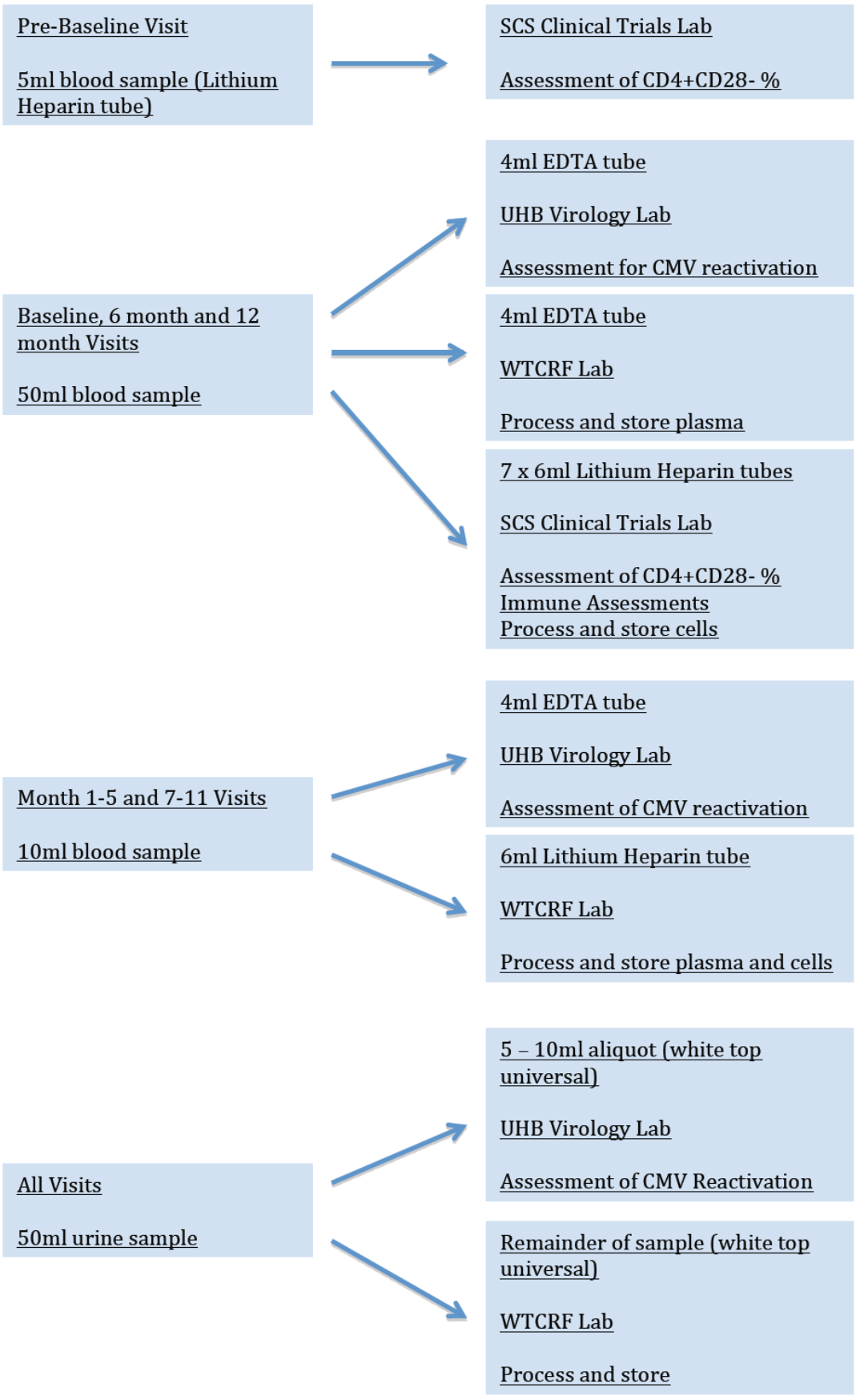
Pre-Baseline Visit
5ml blood sample

Randomisation
PC-CRTU

Treatment Arm	Control Arm
Baseline visit 50ml blood sample Urine sample Safety assessment and blood tests * Pulse wave velocity measurements	Baseline visit 50ml blood sample Urine sample Safety assessment Pulse wave velocity measurements
Month 1 – Month 5 Visit 10ml blood sample Urine sample Safety assessment and blood tests *	Month 1 – Month 5 Visit 10ml blood sample Urine sample Safety assessment
Month 6 Visit 50ml blood sample Urine sample Safety assessment and blood tests * Pulse wave velocity measurements	Month 6 Visit 50ml blood sample Urine sample Safety assessment Pulse wave velocity measurements
Month 7 Visit 10ml blood sample Urine sample Safety assessment and blood tests *	Month 7 Visit 10ml blood sample Urine sample Safety assessment
Month 8 – Month 11 Visit 10ml blood sample Urine sample Safety assessment	Month 8 – Month 11 Visit 10ml blood sample Urine sample Safety assessment
Month 12 Visit 50ml blood sample Urine sample Safety assessment Pulse wave velocity measurements	Month 12 Visit 50ml blood sample Urine sample Safety assessment Pulse wave velocity measurements

* Safety blood tests as defined in Section 7.4

Sample Flow Diagram (Aim 2)



4.3 Outcome Measures

Primary Outcome

- Proportion of patients with CMV reactivation, as assessed by measurable viral load on quantitative blood or urine PCR.

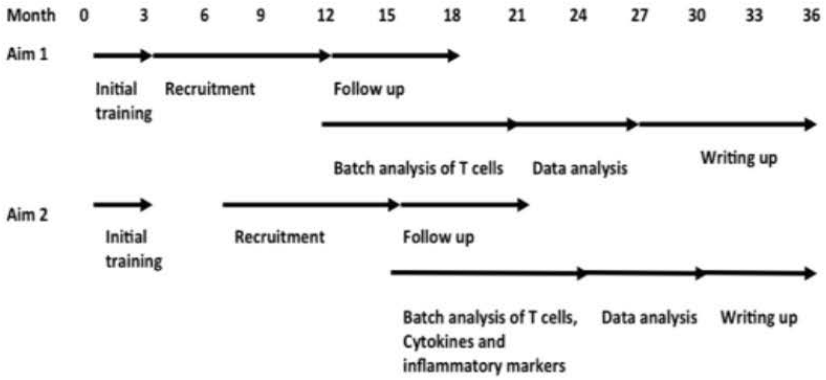
Secondary Outcome

- Safety
- Change in immune phenotype of the CD4+ CMV-specific T-cell population at 6 months (i.e. CD4+CD28- T-cell percentage)
- Change in markers of inflammation including serum concentrations of pro and anti-inflammatory cytokines (TNF-α, IFN-γ, IL-2, IL-6, IL-10, IL-17) and markers of systemic inflammation (highly sensitive CRP, ESR)

Tertiary Outcome

- Persistence of valaciclovir effect on immune phenotype of CD4+ T-cells at 6 months post treatment (i.e. CD4+CD28- T-cell percentage)

4.4 Study Timeline



5 PARTICIPANT ENTRY

5.1 Recruitment

Patients will be recruited from the Vasculitis Clinic in the Wellcome Trust Clinical Research Facility at University Hospitals Birmingham NHS Foundation Trust. The vasculitis clinic has more than 200 patients under long-term follow up. Recruitment will take place over a period of 1-1.5 years. Approximately 90% of patients are in remission at any one time and 70% are seropositive for CMV. Vasculitis patients are a highly motivated group of individuals with a keen interest in research. They have an excellent record of involvement in clinical trials and we have had excellent recruitment rates to many local and international clinical trials. We anticipate 125 of our patients attending the clinic to be eligible for this study. Recruitment of 40% would achieve our target, this is a conservative recruitment rate given our previous experience.

Clinics occur weekly and patients will be approached at routine clinic visits. Patients may also be contacted via the post by sending them a copy of the Patient Information Sheet (PIS). This will be followed up by telephone communication and an appointment at the Wellcome Trust Clinical Research Facility. Recruitment will be reviewed regularly to ensure targets are being met.

5.2 Pre-Randomisation Evaluations

- Serological typing for the presence of CMV-specific antibody
- Immunological analysis to measure the CMV-specific CD4+CD28- T-cell immune response
- Baseline renal (sodium, potassium, urea, creatinine, glomerular filtration rate (eGFR)) and liver function (albumin, alanine transaminase, alkaline phosphatase, bilirubin) tests; tests performed within 6 months of the pre-baseline visit can be used for this assessment

5.3 Sample Size

Aim 1

60 CMV seropositive ANCA-associated vasculitis patients (Patient Cohort - Aim 1) and 30 healthy controls (Control Cohort - Aim 1)

In addition, a long-term cohort comprised of 20 patients that took part in a study 9 years ago and are currently under clinical follow up at the University of Birmingham Vasculitis Clinic will be identified and recruited

Aim 2 (Clinical Trial)
50 CMV seropositive ANCA-associated vasculitis patients will be randomised to two groups of 25 (Control group will comprise 25 patients from the patient cohort in Aim 1)

Sample size basis for Aim 2
In an "immunocompetent" elderly population CMV reactivation occurred in 90% at 6 months. It is expected that CMV reactivation will be at least this in our immunosuppressed population. Information for dosing regimens using antiviral prophylaxis in renal transplant patients has demonstrated over 90% suppression of CMV reactivation. Our sample size assumes 90% reactivation in the control limb and a conservative estimate of 50% reactivation in the treated group. The estimated sample size is 50 patients, 25 patients in each arm based on 80% power and at a significance level p=0.05 (two sided test).

5.4 Inclusion Criteria

- Documented diagnosis of Wegener's granulomatosis (now called Granulomatosis with Polyangiitis), microscopic polyangiitis or renal limited vasculitis according to Chapel Hill Consensus Conference criteria.
- In stable remission (no documented clinical disease activity) for at least 6 months prior to entry.
- On maintenance immunosuppression with prednisolone, mycophenolate mofetil or azathioprine alone or in combination (maximum 2 agents).
- Documented past evidence (any time point) of CMV infection (CMV-specific immunoglobulin G detected in peripheral blood).
- Documentation that female patients of child bearing potential are not pregnant and using an appropriate form of contraception.
- Written informed consent for study participation

5.5 Exclusion Criteria

- Stage 5 chronic kidney disease (eGFR<15ml/minute/1.73m^2); tests performed within 6 months of the pre-baseline visit can be used for this assessment
- Other significant chronic infection (HIV, HBV, HCV, TB).
- B-cell depleting therapy within 12 months or T-cell depleting therapy within 6 months.
- Treatment with anti-CMV therapies in last month
- Underlying medical conditions, which in the opinion of the Investigator place the patient at unacceptably high risk for participating in the study.
- Inability to fully or appropriately participate in the study.

5.6 Withdrawal Criteria

Subjects will be withdrawn from the trial if they choose not to continue or if the investigators feel that continued participation in the trial is inappropriate. The reason for withdrawal will be captured in the medical notes and the CRF. Subjects who withdraw from the intervention will be asked if they are prepared to continue to attend follow-up clinics. Experience from previous studies is that many people who decide to stop active intervention are still prepared to attend for follow-up. Subjects withdrawing within the first month of treatment may be replaced but not thereafter. Analysis will be by intention to treat.

6 RANDOMISATION PROCEDURE

Randomisation will be undertaken using the Primary Care Clinical Research and Trials Unit's (PC-CRTU, fully accredited by the NIHR as a trials unit) independent telephone based randomisation system. We will employ block randomisation by CD4+CD28- T-cell expansion percentage (cut-off 40%).

7 TREATMENT

7.1 Treatment Arms

Treatment Group – Aim 2: 2000 mg valaciclovir q.d.s

Control Group – Aim 2: No additional treatment

7.2 Dose Modifications for Toxicity

In the event of toxicity (scored using the NCI Common Terminology Criteria for Adverse Events (CTCAE) v4.0 (Appendix)) of grade 2 or less, the adverse event(s) will be discussed with the Principal Investigator or one of the Co-Investigators to determine whether drug administration should be temporarily withdrawn or the dose reduced. In the event of toxicity of grade 3 or more, the adverse event(s) will be discussed with the Principal Investigator. In such a case the expectation will be to withdraw drug administration for one week unless the adverse event(s) is judged by the Principal Investigator to be unrelated to the study drug. Re-introduction will be based on clinical review.

Undesirable effects can be classified as followed, based on frequency:

Very common >1/10
Headache

Common >1/100, <1/10
Nausea and vomiting, diarrhoea
Dizziness
Rashes, pruritus

Uncommon >1/1000, <1/100
Leucopenia, thrombocytopenia (leucopenia mainly in immuno-compromised patients)
Confusion, hallucinations, decreased consciousness, tremor, agitation
Dyspnoea
Abdominal discomfort
Reversible increase in liver function tests
Urticaria
Renal pain

Rare <1:1000, >1/10000
Renal impairment (especially elderly or pre-existing renal impairment on high doses)
Angioedema
Ataxia, dysarthria, convulsions, encephalopathy, coma, psychosis
anaphylaxis

Dose modification according to creatinine clearance (CrCl ml/min) *

- > 75 2g q.d.s.
- 51-75 1.5g q.d.s.
- 26-50 1.5g t.d.s.
- 10-25 1.5g b.d.

* Tests performed within 6 months of the pre-baseline visit can be used for this assessment

7.3 Interactions with Other Drugs

As stated in the BNF, valaciclovir is closely related to aciclovir and will have the following interactions:

- Ciclosporin – increased risk of nephrotoxicity when aciclovir given with ciclosporin
- Mycophenolate – plasma concentration of aciclovir increased by myophenolate, also plasma concentration of inactive metabolite of mycophenolate
- Probenecid – excretion of aciclovir reduced by probenecid (increased plasma concentration)
- Tacrolimus – possible increased risk of nephrotoxicity when aciclovir given with tacrolimus
- Theophylline – aciclovir possibly increases plasma concentration of theophylline

The combination of valaciclovir and nephrotoxic medicines, especially immunosuppressant agents like ciclosporin, tacrolimus and mycophenolate mofetil, should be done carefully and demands regular monitoring which will be achieved by monthly monitoring of full blood count, renal and liver function (see 7.4 below). This also applies to aminoglycosides, platinum compounds, iodinated contrast media, methotrexate, pentamidine and foscarnet.

Aciclovir is eliminated primarily unchanged in the urine via active tubular secretion. Any drugs administered concurrently that compete with this mechanism for elimination (e.g. cimetidine, probenecid and mycophenolate mofetil) may increase aciclovir plasma concentrations following aciclovir administration. In patients receiving high dose aciclovir (8000 mg/day) for CMV prophylaxis, caution is required during concurrent administration with this kind of medicine.

7.4 Monitoring of Renal Function

Renal function will be checked prior to commencing treatment with valaciclovir (tests performed within a 6 month period prior to commencing treatment will be used for this assessment). Thereafter it will be regularly checked at monthly intervals for the duration of the 6 month treatment. Renal function as well as liver function (as defined in 5.2) and a full blood count (haemoglobin, white cell count, platelets, mean cell volume) will also be checked in the case of a serious adverse

event arising in a patient in the interim period between the regular monthly blood tests.

7.5 Dispensing

Valaciclovir hydrochloride 500 mg film coated tablets will be prescribed by the study investigators and dispensed by the University Hospital Birmingham Pharmacy.

8 TRIAL MANAGEMENT

CANVAS will be coordinated by the Trial Management Group (Chief Investigator, Co-Investigators and Statistician) in conjunction with the Wellcome Trust Clinical Research Facility based at University Hospital Birmingham according to the current guidelines for Good Clinical Practice and ensuring protection of patients' rights as detailed in the Declaration of Helsinki (Appendix).

8.1 Roles and Responsibilities

The CI takes overall responsibility for the conduct of the study.

8.2 Trial Steering Committee

The Trial Steering Committee (TSC) will provide the overall supervision of the trial, in particular: trial progress, protocol compliance, patient safety and review of updated information. Part of the role of TSC will be to review safety data after the first 10 patients have completed treatment.

The TSC will include the Trial Management Group as well as two Independent Consultant Nephrologists not involved in the study or regular review of patients recruited in the trial.

8.3 Trial Closure

For the purposes of Clinical Trial Authorisation (CTA), the study end date is deemed to be the last visit of the last subject (LVLS).

For the purposes of Multicentre Research Ethics Committee approval, the study end date is deemed to be the date of last data capture.

9 PHARMACOVIGILANCE

The collection and reporting of data on Adverse Events and Serious Adverse Events will be in accordance with EU Directive 2001/20/EC and UK Legislation.

9.1 Definitions

Adverse Event (AE): any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product, which does not necessarily have a causal relationship with the treatment. *An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporarily associated with use of an investigational medicinal product (IMP), whether or not considered related to the IMP.*

Adverse Reactions (AR): all untoward and unintended responses to an IMP, related to any dose administered. *All AEs judged by the reporting investigator or sponsor as having a reasonable causal relationship to an IMP qualify as adverse reactions. The expression 'reasonable causal relationship' means to convey in general that there is evidence or argument to suggest a causal relationship.*

Unexpected adverse reaction: an AR, the nature or severity of which is not consistent with the applicable product information (e.g. Investigator's Brochure for an unapproved investigational product, or a Summary of Product Characteristics (SmPC) for an authorized product). *When the outcome of the adverse reaction is not consistent with the applicable product information, this adverse reaction should be considered as unexpected. Side-effects documented in the SmPC which occur in a more severe form than anticipated are also considered to be unexpected.*

Serious Adverse Event (SAE) or Serious Adverse Reaction (SAR): any untoward medical occurrence or effect that at any dose:

- Results in death
- Is life-threatening – *an event in which the subject was at risk of death at the time of event, not an event that hypothetically might have caused death if it were more severe*
- Requires hospitalization, or prolongation of existing inpatient's hospitalization
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect

Medical judgment should be exercised in deciding whether an AE/AR is serious in other situations. Important AE/ARs that are not immediately life-threatening or do not result in death or hospitalization but may jeopardise the subject or require intervention to prevent one of the other outcomes listed above, should also be considered serious.

Suspected Unexpected Serious Adverse Reaction (SUSAR): any suspected adverse reaction related to an IMP that is both unexpected and serious.

9.2 Causality

Some AE/ARs that occur in this trial, whether they are serious or not, will be expected treatment-related toxicities due to the drug used in this trial. The assignment of the causality will be made by the Investigators responsible for the care of the participants using the definitions in the table below.

If any doubt about the causality exists, the Investigator will inform the Chief Investigator. Other clinicians within the TSC may be asked to advise in some cases.

In the case of discrepant views on causality between the Investigator and others, all parties will discuss the case. In the event that no agreement is made, the MHRA will be informed of both points of view.

Relationship	Description
Unrelated	There is no evidence of causal relationship
Unlikely	There is little evidence to suggest a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). However the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant treatments).
Possible	There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant treatments).
Probable	There is evidence to suggest a causal relationship and the influence of other factors in unlikely.
Definitely	There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.

9.3 Reporting Procedures

Non-Serious AE/ARs

All such events whether expected or not, should be reported in the toxicity section of the relevant Case Report Form.

Abnormal safety blood laboratory values will not be routinely reported unless they fall outside of the normal range and have changed by greater than 20% from the baseline value as the majority of AAV patients will routinely have abnormal results due to their condition.

SAEs, SARs and SUSARs

An SAE form (Appendix) should be completed and submitted to the Chief Investigator for all SAEs and SARs within 24 hours.

A pre-existing condition should not be reported as an adverse event unless the condition worsens or episodes increase in frequency during the adverse event reporting period.

The Trial Management Group will produce a full report using the SAE form, and report the events to the sponsor, ethics committee and MHRA within the required timelines (fatal and life-threatening SUSARs within 7 days of notification, and non-life-threatening within 15 days). The Chief Investigator's assessment of causality of SAEs (i.e. their relationship to trial treatment) will be reported on the Serious Adverse Event form.

10 ASSESSMENT AND FOLLOW-UP

10.1 Loss to Follow-up

Subjects will be studied for 6 months after recruitment in the case of Aim 1 and 12 months after recruitment (6 months whilst on treatment and 6 months follow-up after discontinuation) in the case of Aim 2. The follow-up cohort in Aim 1 will have a single blood sample drawn with no requirement for follow up.

If participants are lost to follow up we may choose to recruit additional subjects to achieve the required total number of participants in each group (60 in each group for Aim 1 and 25 in each group for Aim 2). The decision will depend on timing of loss to follow-up and the corresponding value of the information gained from the subject.

11 STATISTICS AND DATA ANALYSIS

11.1 Statistics and Data Analysis Plan

The primary outcome measure for therapeutic efficacy will be the proportion of patients reactivating CMV.

All analyses will be performed using the intention to treat principle.

Baseline covariates will be compared between the two arms to observe balance and the success of randomisation.

The primary analysis will test the hypothesis that there is no difference in CMV reactivation between those CMV seropositive patients receiving antiviral prophylaxis compared with those receiving usual treatment.

The primary analysis will be performed unadjusted, and repeated adjusted for baseline covariates using logistic regression. Differences in treatments will be determined using Cox proportional-hazards regression analysis. Values of $p<0.05$ will be considered statistically significant.

Secondary end-points will be analysed using parametric and non-parametric tests as appropriate. For secondary outcomes comparisons will be made between groups using absolute measures of immune function and change between data at entry and end of treatment within groups.

A safety analysis will be performed on all treated patients. The number of events and incidence of adverse events by system organ class will be summarized and relationship to treatment noted. Initial safety analyses will be performed after 10 patients have completed treatment.

11.2 Data Handling, Record Keeping and Retention

Identifiable data will be retained for the study period and sufficient time after in order to inform participants regarding the results. All analyses will be undertaken on anonymised datasets with study identifiers replacing personal data. All staff have confidentiality clauses in their honorary and substantive contracts.

All personal details will be kept on password protected servers within UHB NHS Trust. In order to analyse data anonymised data will be transferred to password protected servers in the University of Birmingham. No data will be stored on computer hard drives.

11.3 Data Access and Quality Assurance

Storage of personal data on manual files paper: Copies of the consent form will be stored at the Wellcome Trust Clinical Research Facility (WTCRF), UHB NHS Trust in locked filing cabinets, in rooms which will be locked when unoccupied. Access to the WTCRF is by authorized swipe card access only.

Storage of data on university computers: All personal details will be kept on password protected servers within UHB NHS Trust. In order to analyse data anonymised data will be transferred to password protected servers in the University of Birmingham. No data will be stored on computer hard drives.

Analysed data will be stored in the form of anonymised datasets in password protected, access limited electronic format. The Principal Investigator and Co-Investigators will have access to this data. In the event of this data being considered for use in a follow up associated study further informed consent will be sought from the individual. This will be stated in the patient information sheet for the present study.

11.4 Case Report Forms

Case Report Forms will contain information on:

- Eligibility
- Randomisation
- On-study details
- On treatment follow up details
- Sample analysis details
- Adverse Events / Serious Adverse Events details
- Concomitant medication details

There will be direct source data entry onto CRF pages that contain adverse event / serious adverse event and concomitant medication details.

12 MONITORING

12.1 Risk Assessment

The study has undergone extensive independent external review. It has received peer review by the Wellcome Trust as part of a Training Fellowship Application. The outcome was successful.

The study has also been peer reviewed by Vasculitis UK and was successful in receiving additional funding from this organization.

Valaciclovir and its related drug aciclovir have been used in many millions of individuals over the last 20 years. Their safety record is impressive and aciclovir has been considered for 'over the counter' status in the USA. The dose of valaciclovir utlilised in this study is within that currently licensed within the UK.

13 REGULATORY ISSUES

13.1 Clinical Trial Authorization

The study will be carried out under a Clinical Trial Authorisation (CTA) from the UK competent authority, MHRA, and conducted in accordance with EU directive 2001/20/EC and UK legislation.

13.2 Ethics Approval

The study will not commence until ethical approval has been obtained from the Local Research Ethics Committee via an application to the Integrated Research Application System (IRAS).

The trial will be conducted in accordance with the recommendations for physicians involved in research of human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions (Appendix).

13.3 Patient Consent

Participants will be identified from the Vasculitis Clinic at the Wellcome Trust Clinical Research Facility at University Hospital Birmingham. Informed consent using the 'Consent Form' (Appendix) will be obtained by a member of the research team. A Patient Information Sheet (Appendix) will be given to each potential participant prior to recruitment and the risks and benefits of participating in the study will be clearly explained. The patient will be given ample time to read the information sheet and the opportunity to enquire about details on the study. All questions or concerns should be answered to the satisfaction of the patient. Sufficient time will be allowed (at least 24 hours) for individuals to make a decision. It will be explained that they are free to refuse to take part and will be informed about their right to withdraw from the trial at any time. If the individual agrees to take part in the study they will be asked to sign and date the Consent Form that will also be signed and dated by the Investigator. A copy of the Consent Form will be given to the patient, a copy filed in the hospital notes and a copy filed in the Investigator file. Throughout the study the individual should have the opportunity to ask questions about the trial and any new information that may be relevant to the patient's willingness to continue participation in the trial should be shared with the patient in a timely manner.

13.4 Confidentiality

The personal data recorded on all documents will be regarded as strictly confidential and will be handled and stored in accordance with the 1998 Data Protection Act as also detailed in Section 11.2 and 11.3.

13.5 Indemnity

Arrangements for insurance and/or indemnity to meet the potential legal liability of the sponsor for harm to participants arising from the management and design of the research: The University of Birmingham has in force a Public Liability Policy and/or Clinical Trials policy which provides cover for claims for negligent harm and the activities here are included within that coverage.

Arrangements for insurance and/or indemnity to meet the potential legal liability of investigators arising from harm to participants in the conduct the research: NHS indemnity scheme or professional indemnity will apply.

13.6 Sponsor

The University of Birmingham will act as the sponsor of this study.

13.7 Funding

The Wellcome Trust is funding the majority of this study as part of a Research Training Fellowship Grant.

The cost of the study drug is funded by Vasculitis UK.

13.8 Audits

The study may be subject to inspection and audit by the MHRA and the University of Birmingham under their remit as sponsor and other regulatory bodies, to ensure adherence to GCP and regulations.

14 FINANCIAL ARRANGEMENTS

14.1 Participant Payments

Participants will be reimbursed for travel expenses where required.

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Appendix A

CHIEF INVESTIGATOR SIGNATURE

I have read and agree to the protocol, as detailed in this document. I agree to adhere to the protocol as outlined and agree that any suggested changes to the protocol must be approved by the Trial Management Group prior to seeking approval from the Independent Ethics Committee.

I am aware of my responsibilities as an Investigator under the guidelines of Good Clinical Practice (GCP), the declaration of Helsinki, local regulations (as applicable) and the study protocol and I agree to conduct the study according to these guidelines and to appropriately direct and assist the staff under my control, who will be involved in the study.

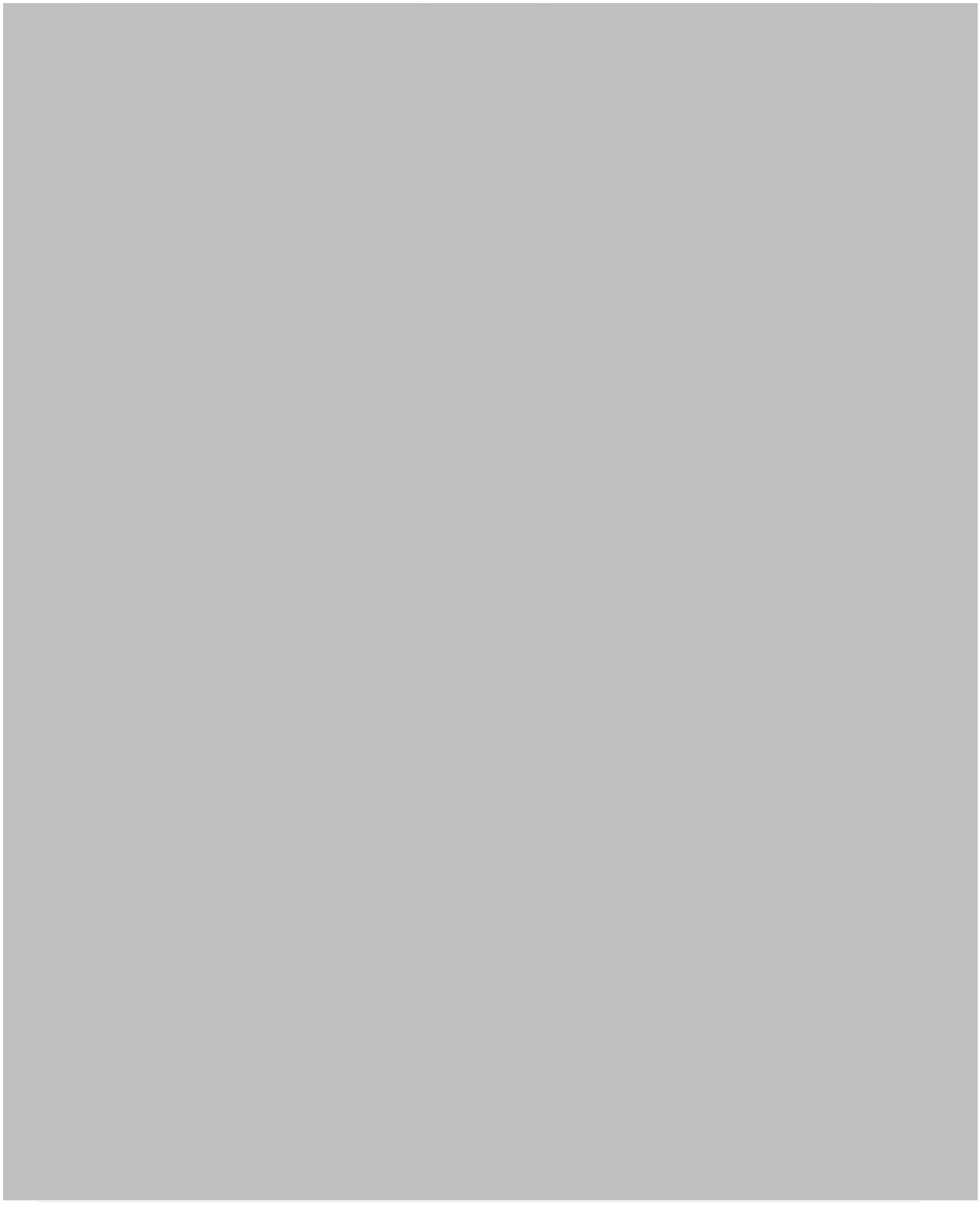
Professor Lorraine Harper

Signature:……

Date:……

Appendix B

Signed Letter of Sponsorship Approval



Appendix C

NIC Common Terminology Criteria for Adverse Events
(CTCAE) Version 4.0

Toxicities will be recorded according to the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

The full CTCAE document is available for download on the National Cancer Institute (NCI) website at the following address:

http://ctep.cancer.gov/reporting/ctc.html

Appendix D

WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI
Ethical Principles for Medical Research Involving Human Subjects

Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the: 29th WMA General Assembly, Tokyo, Japan, October 1975 35th WMA General Assembly, Venice, Italy, October 1983 41st WMA General Assembly, Hong Kong, September 1989 48th WMA General Assembly, Somerset West, Republic of South Africa, October 1996 52nd WMA General Assembly, Edinburgh, Scotland, October 2000 53rd WMA General Assembly, Washington 2002 (Note of Clarification on paragraph 29 added) 55th WMA General Assembly, Tokyo 2004 (Note of Clarification on Paragraph 30 added) 59th WMA General Assembly, Seoul, October 2008

A. INTRODUCTION

1. The World Medical Association (WMA) has developed the Declaration of Helsinki as a statement of ethical principles for medical research involving human subjects, including research on identifiable human material and data.
The Declaration is intended to be read as a whole and each of its constituent paragraphs should not be applied without consideration of all other relevant paragraphs.
2. Although the Declaration is addressed primarily to physicians, the WMA encourages other participants in medical research involving human subjects to adopt these principles.
3. It is the duty of the physician to promote and safeguard the health of patients, including those who are involved in medical research. The physician's knowledge and conscience are dedicated to the fulfilment of this duty.
4. The Declaration of Geneva of the WMA binds the physician with the words, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act in the patient's best interest when providing medical care."
5. Medical progress is based on research that ultimately must include studies involving human subjects. Populations that are underrepresented in medical research should be provided appropriate access to participation in research.
6. In medical research involving human subjects, the well-being of the individual research subject must take precedence over all other interests.
7. The primary purpose of medical research involving human subjects is to understand the causes, development and effects of diseases and improve preventive, diagnostic and therapeutic interventions (methods, procedures and treatments). Even the best current interventions must be evaluated continually through research for their safety, effectiveness, efficiency, accessibility and quality.
8. In medical practice and in medical research, most interventions involve risks and burdens.
19. Medical research is subject to ethical standards that promote respect for all human subjects and protect their health and rights. Some research populations are particularly vulnerable and need special protection. These include those who cannot give or refuse consent for themselves and those who may be vulnerable to coercion or undue influence.
10. Physicians should consider the ethical, legal and regulatory norms and standards

for research involving human subjects in their own countries as well as applicable international norms and standards. No national or international ethical, legal or regulatory requirement should reduce or eliminate any of the protections for research subjects set forth in this Declaration.

B. PRINCIPLES FOR ALL MEDICAL RESEARCH

11. It is the duty of physicians who participate in medical research to protect the life, health, dignity, integrity, right to self-determination, privacy, and confidentiality of personal information of research subjects.

12. Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and adequate laboratory and, as appropriate, animal experimentation. The welfare of animals used for research must be respected.

13. Appropriate caution must be exercised in the conduct of medical research that may harm the environment.

14. The design and performance of each research study involving human subjects must be clearly described in a research protocol. The protocol should contain a statement of the ethical considerations involved and should indicate how the principles in this Declaration have been addressed. The protocol should include information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest, incentives for subjects and provisions for treating and/or compensating subjects who are harmed as a consequence of participation in the research study. The protocol should describe arrangements for post-study access by study subjects to interventions identified as beneficial in the study or access to other appropriate care or benefits.

15. The research protocol must be submitted for consideration, comment, guidance and approval to a research ethics committee before the study begins. This committee must be independent of the researcher, the sponsor and any other undue influence. It must take into consideration the laws and regulations of the country or countries in which the research is to be performed as well as applicable international norms and standards but these must not be allowed to reduce or eliminate any of the protections for research subjects set forth in this Declaration. The committee must have the right to monitor ongoing studies. The researcher must provide monitoring information to the committee, especially information about any serious adverse events. No change to the protocol may be made without consideration and approval by the committee.

16. Medical research involving human subjects must be conducted only by individuals with the appropriate scientific training and qualifications. Research on patients or healthy volunteers requires the supervision of a competent and appropriately qualified physician or other health care professional. The responsibility for the protection of research subjects must always rest with the physician or other health care professional and never the research subjects, even though they have given consent.

17. Medical research involving a disadvantaged or vulnerable population or community is only justified if the research is responsive to the health needs and priorities of this population or community and if there is a reasonable likelihood that this population or community stands to benefit from the results of the research.

18. Every medical research study involving human subjects must be preceded by careful assessment of predictable risks and burdens to the individuals and communities involved in the research in comparison with foreseeable benefits to them and to other individuals or communities affected by the condition under investigation.

19. Every clinical trial must be registered in a publicly accessible database before recruitment of the first subject.
20. Physicians may not participate in a research study involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians must immediately stop a study when the risks are found to outweigh the potential benefits or when there is conclusive proof of positive and beneficial results.
21. Medical research involving human subjects may only be conducted if the importance of the objective outweighs the inherent risks and burdens to the research subjects.
22. Participation by competent individuals as subjects in medical research must be voluntary. Although it may be appropriate to consult family members or community leaders, no competent individual may be enrolled in a research study unless he or she freely agrees.
23. Every precaution must be taken to protect the privacy of research subjects and the confidentiality of their personal information and to minimize the impact of the study on their physical, mental and social integrity.
24. In medical research involving competent human subjects, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail, and any other relevant aspects of the study. The potential subject must be informed of the right to refuse to participate in the study or to withdraw consent to participate at any time without reprisal. Special attention should be given to the specific information needs of individual potential subjects as well as to the methods used to deliver the information. After ensuring that the potential subject has understood the information, the physician or another appropriately qualified individual must then seek the potential subject's freely-given informed consent, preferably in writing. If the consent cannot be expressed in writing, the non-written consent must be formally documented and witnessed.
25. For medical research using identifiable human material or data, physicians must normally seek consent for the collection, analysis, storage and/or reuse. There may be situations where consent would be impossible or impractical to obtain for such research or would pose a threat to the validity of the research. In such situations the research may be done only after consideration and approval of a research ethics committee.
26. When seeking informed consent for participation in a research study the physician should be particularly cautious if the potential subject is in a dependent relationship with the physician or may consent under duress. In such situations the informed consent should be sought by an appropriately qualified individual who is completely independent of this relationship.
27. For a potential research subject who is incompetent, the physician must seek informed consent from the legally authorized representative. These individuals must not be included in a research study that has no likelihood of benefit for them unless it is intended to promote the health of the population represented by the potential subject, the research cannot instead be performed with competent persons, and the research entails only minimal risk and minimal burden.
28. When a potential research subject who is deemed incompetent is able to give assent to decisions about participation in research, the physician must seek that assent in addition to the consent of the legally authorized representative. The potential

subject's dissent should be respected.

29. Research involving subjects who are physically or mentally incapable of giving consent, for example, unconscious patients, may be done only if the physical or mental condition that prevents giving informed consent is a necessary characteristic of the research population. In such circumstances the physician should seek informed consent from the legally authorized representative. If no such representative is available and if the research cannot be delayed, the study may proceed without informed consent provided that the specific reasons for involving subjects with a condition that renders them unable to give informed consent have been stated in the research protocol and the study has been approved by a research ethics committee. Consent to remain in the research should be obtained as soon as possible from the subject or a legally authorized representative.

30. Authors, editors and publishers all have ethical obligations with regard to the publication of the results of research. Authors have a duty to make publicly available the results of their research on human subjects and are accountable for the completeness and accuracy of their reports. They should adhere to accepted guidelines for ethical reporting. Negative and inconclusive as well as positive results should be published or otherwise made publicly available. Sources of funding, institutional affiliations and conflicts of interest should be declared in the publication. Reports of research not in accordance with the principles of this Declaration should not be accepted for publication.

C. ADDITIONAL PRINCIPLES FOR MEDICAL RESEARCH COMBINED WITH MEDICAL CARE

31. The physician may combine medical research with medical care only to the extent that the research is justified by its potential preventive, diagnostic or therapeutic value and if the physician has good reason to believe that participation in the research study will not adversely affect the health of the patients who serve as research subjects.

32. The benefits, risks, burdens and effectiveness of a new intervention must be tested against those of the best current proven intervention, except in the following circumstances:

• •

The use of placebo, or no treatment, is acceptable in studies where no current proven intervention exists; or Where for compelling and scientifically sound methodological reasons the use of placebo is necessary to determine the efficacy or safety of an intervention and the patients who receive placebo or no treatment will not be subject to any risk of serious or irreversible harm. Extreme care must be taken to avoid abuse of this option.

33. At the conclusion of the study, patients entered into the study are entitled to be informed about the outcome of the study and to share any benefits that result from it, for example, access to interventions identified as beneficial in the study or to other appropriate care or benefits.

34. The physician must fully inform the patient which aspects of the care are related to the research. The refusal of a patient to participate in a study or the patient's decision to withdraw from the study must never interfere with the patient-physician relationship.

35. In the treatment of a patient, where proven interventions do not exist or have been ineffective, the physician, after seeking expert advice, with informed consent from the patient or a legally authorized representative, may use an unproven

intervention if in the physician's judgment it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, this intervention should be made the object of research, designed to evaluate its safety and efficacy. In all cases, new information should be recorded and, where appropriate, made publicly available.

Appendix E

Pharmacovigilance Logarithm

1. Adverse Event Observed

2. Is it serious? (As per definitions set out in Section 9.1)
   If No → Go to 3
   If Yes → Go to 4

3. It is an AE
   It does not require expedited reporting and can be described on CRF

4. It is an SAE/SAR
   Assess for severity and causality; complete the SAE form and report to the CI within 24 hours
   To go on DSUR
   ➔ Go to 5

5. If it is reasonably causally related to the trial intervention or medication, CI to assess expectedness
   Is it expected? (i.e. included in SmPC)
   If Yes → It is an SAR – To go on DSUR
   If No → It is a SUSAR

   Required expedited reporting to MHRA and REC → Go to 6
   To go on DSUR

6. Is it life threatening or fatal?
   If No → Go to 7
   If Yes → Go to 8

7. Report to MHRA, REC and University of Birmingham Research Governance Team within 15 days

8. Report to MHRA, REC and University of Birmingham Research Governance Team within 7 days

Appendix F

Serious Adverse Event Reporting Form

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS Foundation Trust

CMV Modulation in ANCA-Associated VASculitis (CANVAS)

SERIOUS ADVERSE EVENT REPORTING FORM

Patient ID:

Date of Birth:

Patient Initials:

1. SAE Onset Date: _____ (dd/mm/yyyy)
2. SAE Stop Date: _____(dd/mm/yyyy)
3. Location of serious adverse event: _____
4. Was this an unexpected adverse event? Yes ☐ No ☐
5. Brief description of the nature of the serious adverse event (attach description if more space needed):

   _____

   _____

6. Category of the serious adverse event:

   ☐ death – date __/___/___(dd/mmm/yyyy)
   ☐ life-threatening
   ☐ hospitalization-initial or prolonged
   ☐ disability / incapacity
   ☐ congenital anomaly / birth defect
   ☐ required intervention to prevent permanent impairment
   ☐ other:_____

7. Trial treatment patient was receiving when SAE started:_____
8. Total daily dose:_____
9. Was the trial treatment given at full protocol dose, prior to the SAE?

   _____

10. Date of last trial treatment given, prior to SAE:_____
11. Causal relationship to event:

    ☐ Definitely
    ☐ Probably
    ☐ Possibly
    ☐ Unlikely
    ☐ Not related
    ☐ Not applicable (Control arm – no treatment)

2. Was study intervention discontinued due to event? ☐ Yes ☐ No
3. Other treatments at time of event (include concomitant medication, radiotherapy, surgery, palliative care; exclude any therapy given for the management of SAE)

_____
_____
_____
_____

4. What medications or other steps were taken to treat serious adverse event?

_____

5. Other relevant information to facilitate assessment (Include medical history, drug or alcohol abuse, family history, findings from special investigations, etc.)

_____
_____

6. Was this event expected in view of the patient's clinical history? ☐ Yes ☐ No

7. Additional information

_____
_____
_____

8. Type of report:

☐ Initial
☐ Follow-up
☐ Final

TO BE COMPLETED BY CHIEF INVESTIGATOR

Was the event drug related? ☐ *Yes* ☐ *No*
Was the event unexpected? ☐ *Yes* ☐ *No*
Was the event a SUSAR? ☐ *Yes* ☐ *No*

Comments

_____
_____

*Date reported to MHRA*_____

Signature of Chief Investigator: _____ *Date:* _____

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Appendix 3 Participant Information Sheet and Informed Consent Form

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS
NHS Foundation Trust

CANVAS

CMV modulation of the immune system in **AN**CA-associated **VAS**culitis

Patient (Active) Information Sheet

We would like to invite you to take part in a research study. Before you decide whether or not you wish to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. It explains why we are undertaking this study, what your involvement would be and hopefully answers any questions that you may have at this stage.

What is the purpose of the study?
Cytomegalovirus (CMV) is a common virus that tends not to cause symptoms in healthy individuals. It is present in a large proportion of the population such that by middle age over half the population have had CMV infection. Research has shown that following initial infection the virus remains in the body for life and undergoes periods of reactivation. The immune system normally keeps the virus under control. However this means that a large proportion of T-cells, a part of the immune system important for fighting infection, are 'committed' to controlling CMV. Eventually some of these committed T-cells become "exhausted" and do not appear to work well. The presence of large numbers of "exhausted" immune system cells in the body may impair the ability to control CMV and fight other infections. In most studies this only appears to be a problem very late in life.

However in previous studies in Birmingham we have shown that vasculitis patients who have CMV infection and a lot of "exhausted" cells seem to be more prone to infections. This is important as the treatment for vasculitis also suppresses immune system activity and increases the risk of infection. In addition, CMV infection has recently been linked to increased stiffness of the blood vessels. Increased stiffness of the blood vessels is a risk factor for heart disease and stroke.

There are two main parts to this study.
Firstly we want to further investigate the nature of CMV committed T-cells and "exhausted" cells in patients and healthy people who have CMV infection.
Secondly we want to find out if using medication (Valaciclovir) that blocks CMV activity in the body may allow the immune system and the "exhausted" cells to recover which should then improve the body's ability to fight other infections as well.

Why have I been invited?
You have been invited to participate because you have stable vasculitis disease and are positive for previous CMV infection.

Do I have to take part?
It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. If you decide to participate we will ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?
If you agree to take part we will ask you to provide an initial 5ml blood sample in order to determine the percentage of a particular subset of T-cells linked to CMV. We will then ask you to provide a 50ml (3 tablespoons) blood and urine sample so that we can measure the number of CMV 'committed' T-cells in your blood and confirm that you have had previous CMV infection. We would also want to store some of the cells frozen in liquid nitrogen for use at a later date, as we cannot run all the tests on the day you come to clinic. Additionally, we would like to save some of the blood and urine sample to measure other inflammatory chemicals and proteins and response to other viruses.

Following initial assessment you will be randomly assigned (like tossing a coin) to either continue with your regular vasculitis treatment OR receive additional treatment with an antiviral drug called valaciclovir. Treatment will be in the form of tablet therapy four times a day. There is no need for hospital admission in order to commence treatment. The medication will need to be taken every day for the prescribed length of time (6 months). We will make an appointment for you to be assessed by the research team during a brief hospital visit on a monthly basis for 12 months following commencement of your participation in the study. At each monthly visit we will also ask your permission to collect a further 10ml blood and urine sample to assess how active CMV is in your body as well as a 50ml blood and urine sample at 6 months and 12 months. If you have been assigned to take the antiviral drug we will also check your well-being at each monthly visit.
We would also like to measure your blood pressure as well as the stiffness of your blood vessels at the initial visit, 6 months and 12 months. The equipment used is very similar to a blood pressure machine and the cuffs can be placed over clothing. There are no risks to this procedure, although the inflated cuff can sometimes exert discomfort on the arm.

What are the possible disadvantages and risks of taking part?
All drugs have side effects, and no drug is without risk. However, the drug used in this study is a licensed medication and the research team will be able to adjust the dose should you suffer any side effects.

Common side effects experienced by between 1 in 10 and 1 in 100 patients include headache and nausea. Uncommon side effects experienced by between 1 in 100 and 1 in 1000 patients are shortness of breath and rashes or sensitivity to light. Rare side effects experienced by between 1 in 1,000 and 1 in 10,000 patients include dizziness, confusion, hallucinations, drowsiness, itching, kidney problems, stomach pains, vomiting and diarrhoea. Finally, very rare side effects experienced by less than 1 in 10,000 patients may include allergic reactions, anaemia, inflammation of the liver, acute kidney failure, agitation, tremors and psychosis.

What are the possible benefits of taking part?
There is no immediate benefit in participating in this study. By improving our understanding of the way CMV affects the immune system in vasculitis and determining whether treatment of CMV ameliorates these effects we may be able to improve therapy for vasculitis patients and other patient groups in the future.

What will happen if I don't want to carry on with the study?
You are free to withdraw from the study at any time and this will not affect your care. You can either withdraw completely or choose to keep in contact with us to let us know your progress. Information collected earlier in the study may still be used.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researcher (see contact details) who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure (or a private institution). Details can be obtained from the hospital.

Will my taking part in the study be kept confidential?
All information collected about you during the course of the research will be kept strictly confidential, and any information about you that leaves the hospital/clinical laboratory will have your name and address removed so that you cannot be recognised.

Involvement of the General Practitioner/Family doctor (GP)
Your GP will be informed of your participation in this study in order to monitor for any side-effects associated with the study medication.

What will happen to any samples I give?
Blood samples that you give will be sent to the University Hospital Birmingham Virology Laboratory and our research laboratories (Wellcome Trust Clinical Research Facility (WTCRF) and University of Birmingham Laboratories). Some tests will be done on your blood samples shortly after the samples have been taken. The remaining sample will then be placed in a freezer (WTCRF and University of Birmingham), as other tests will be done some time later. The cells will not be used for financial profit. We would like your permission to retain any cells left at the end of the project for use in future projects (subject to approval by the Research Ethics Committee).

Will any genetic tests be done?
The only genetic test performed will be an assessment of your HLA type (a marker on the surface of white blood cells) as this is essential in carrying out part of the T-cell analysis. This will not produce any findings of clinical significance for yourself or any of your relatives.

What will happen to the results of the research study?
The overall broad scientific results of the study will be published in medical/scientific journals and the publications made available to all participants. You will not be identified in any report or publication.

Who is organising and funding the research?
The study is being organised by the University of Birmingham. The research is being funded by the

Wellcome Trust and the Vasculitis UK (formerly Stuart Strange Trust) patient support group.

Who has reviewed the study?
All research in the NHS is reviewed by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed by the NRES Committee York and Humber - Leeds West Research Ethics Committee and has been given a favourable opinion. The scientific rigour of this study has been reviewed by the Wellcome Trust who have agreed to fund this research. The study has also been reviewed by the trustees of Vasculitis UK who think this is an important study for vasculitis patients.

Further information and contact details
For further information, please contact:
Professor Lorraine Harper, Professor Paul Moss, Dr Matthew Morgan or Dr Dimitrios Chanouzas via the Wellcome Trust Clinical Research Facility at the University Hospitals Birmingham NHS Foundation Trust. Telephone: 0121 371 3170.

Thank you for taking time to consider participating in this study.

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VASCULITIS UK

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS
NHS Foundation Trust

Name:

Date of Birth: Hospital number:

Subject Identification Number for this study:

Patient active consent form
Title of Project: **C**MV modulation of the immune system in **AN**CA-associated **VAS**culitis (CANVAS)
Name of Researcher: Professor Lorraine Harper

Please initial boxes

1. I confirm that I have read and understood the Patient Information Sheet (Version 1.4) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the University of Birmingham, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I understand that my GP will be informed of my participation in this study and may be contacted to provide information about my progress, in confidence, to the organisers. ☐

5. I understand participation is a 12-month commitment. I agree to attend clinics and donate blood and urine samples for study as detailed in the information sheet. ☐

6. I agree that my samples may be stored within the University of Birmingham for future research, providing ethical approval for these additional studies has first been obtained. ☐

7. I also understand that data collected about me for this study is covered under the Data Protection Act 1998 and stored electronically in a secure encoded format. ☐

8. I agree to take part in the above study. ☐

Name of Patient	Date	Signature

Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

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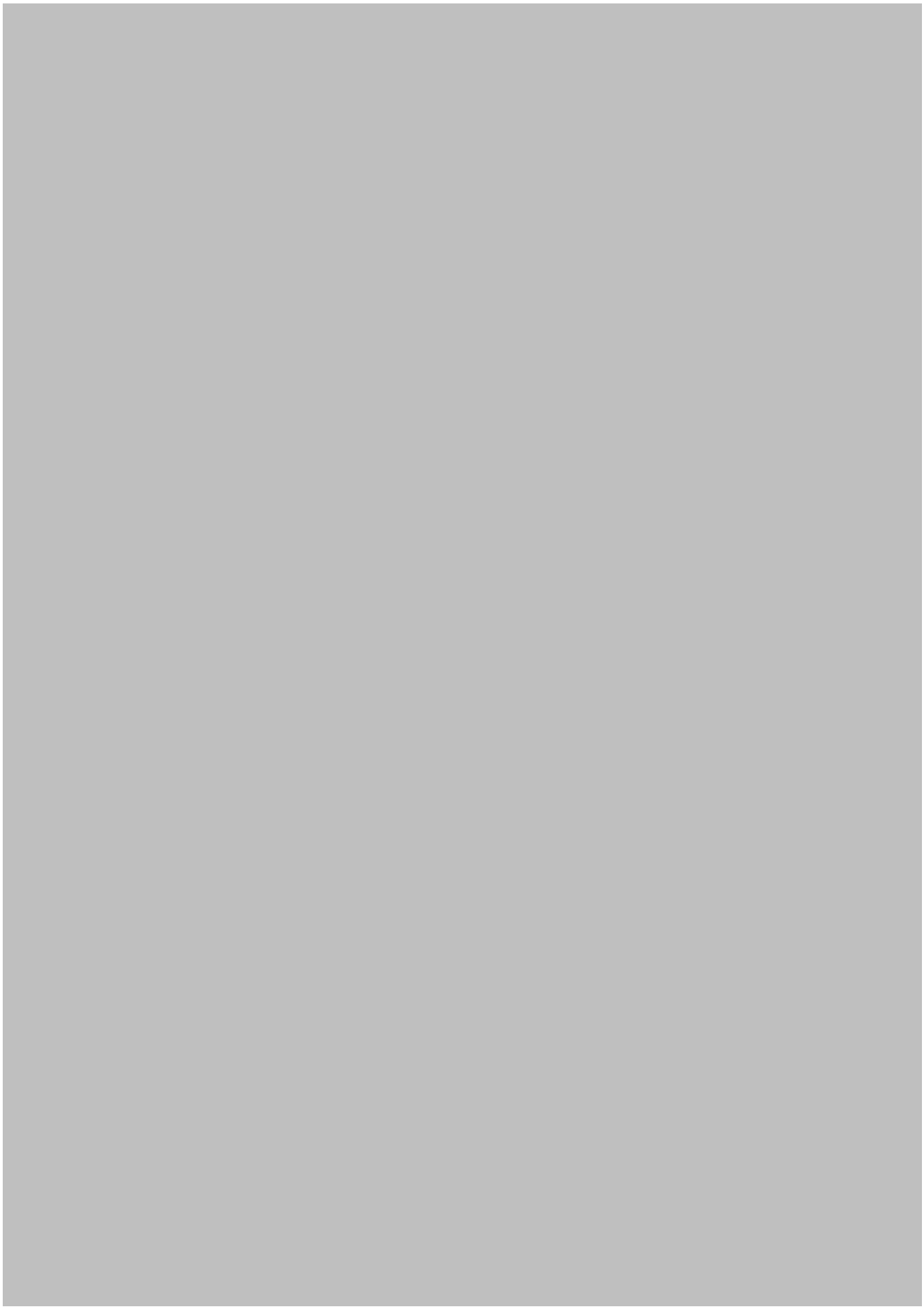
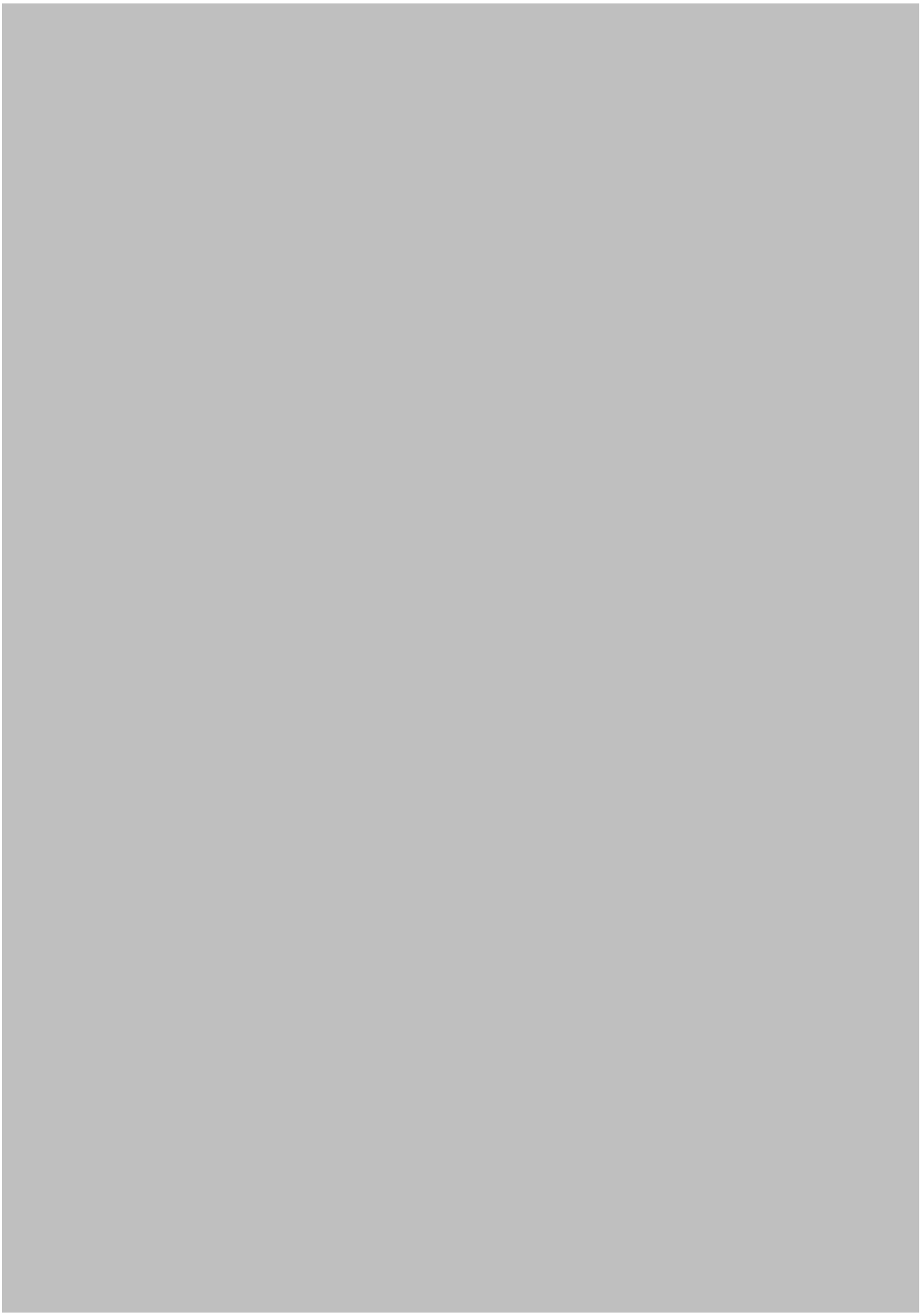
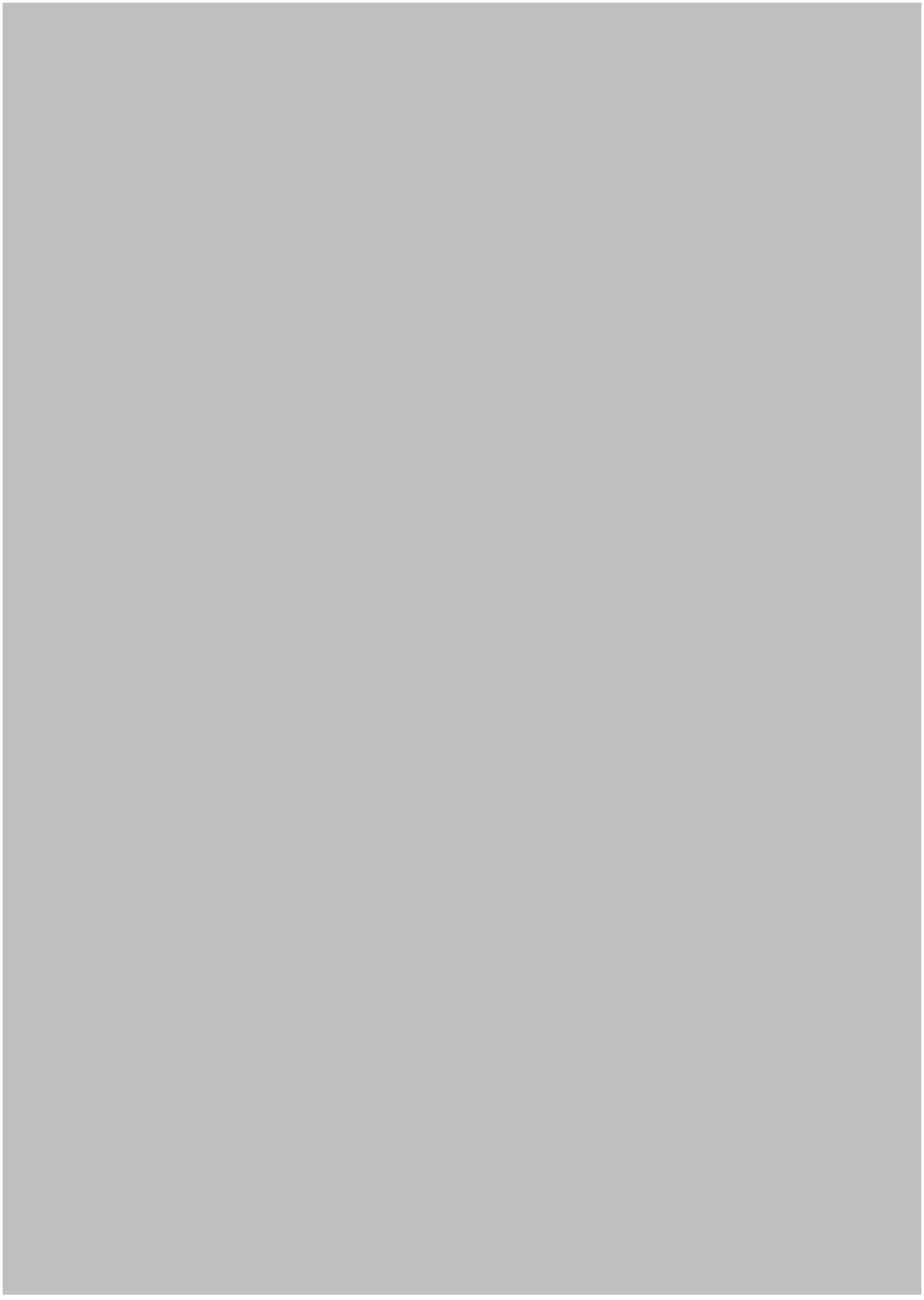
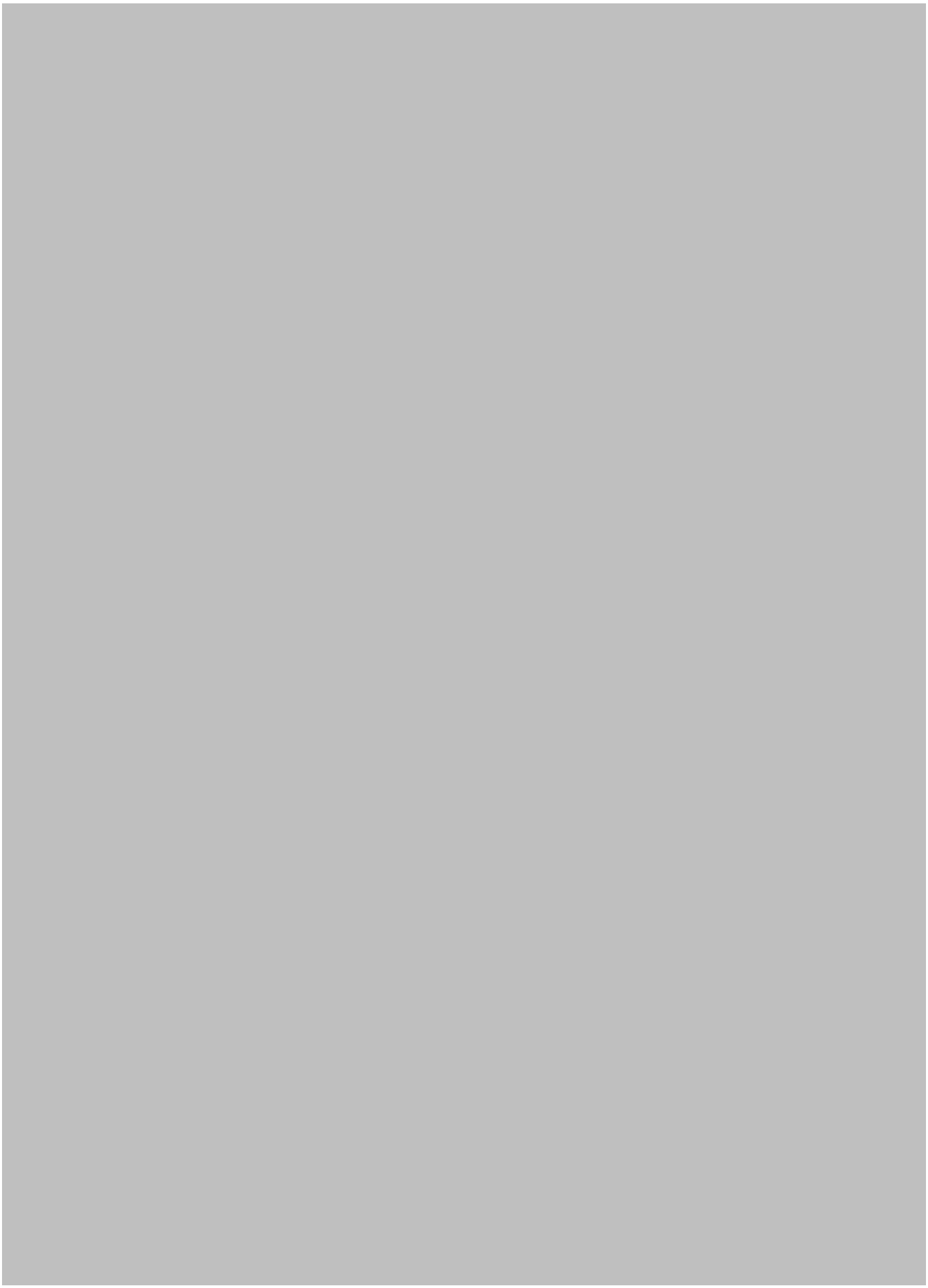
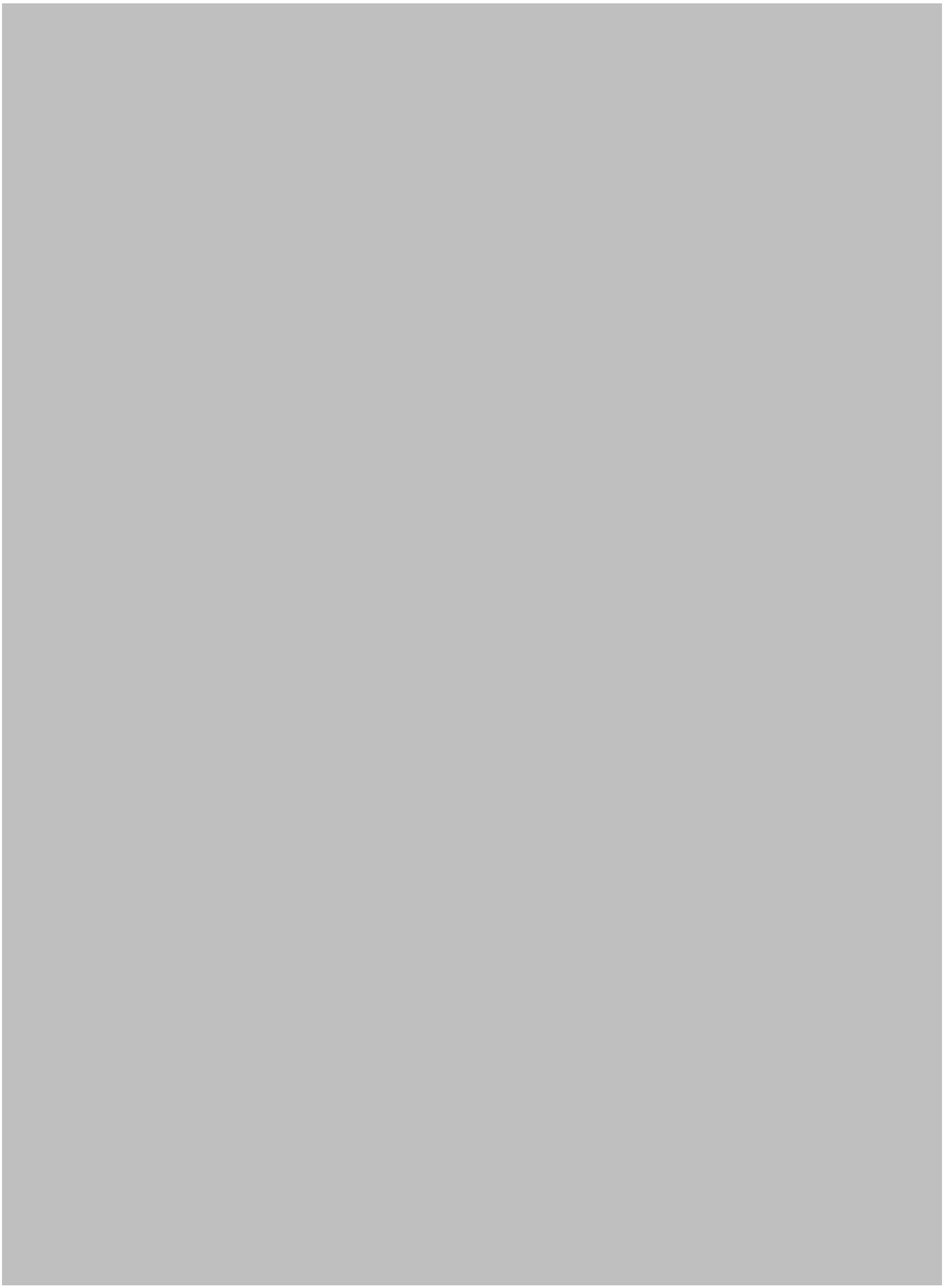
Appendix 4 Vicorder Work Instruction

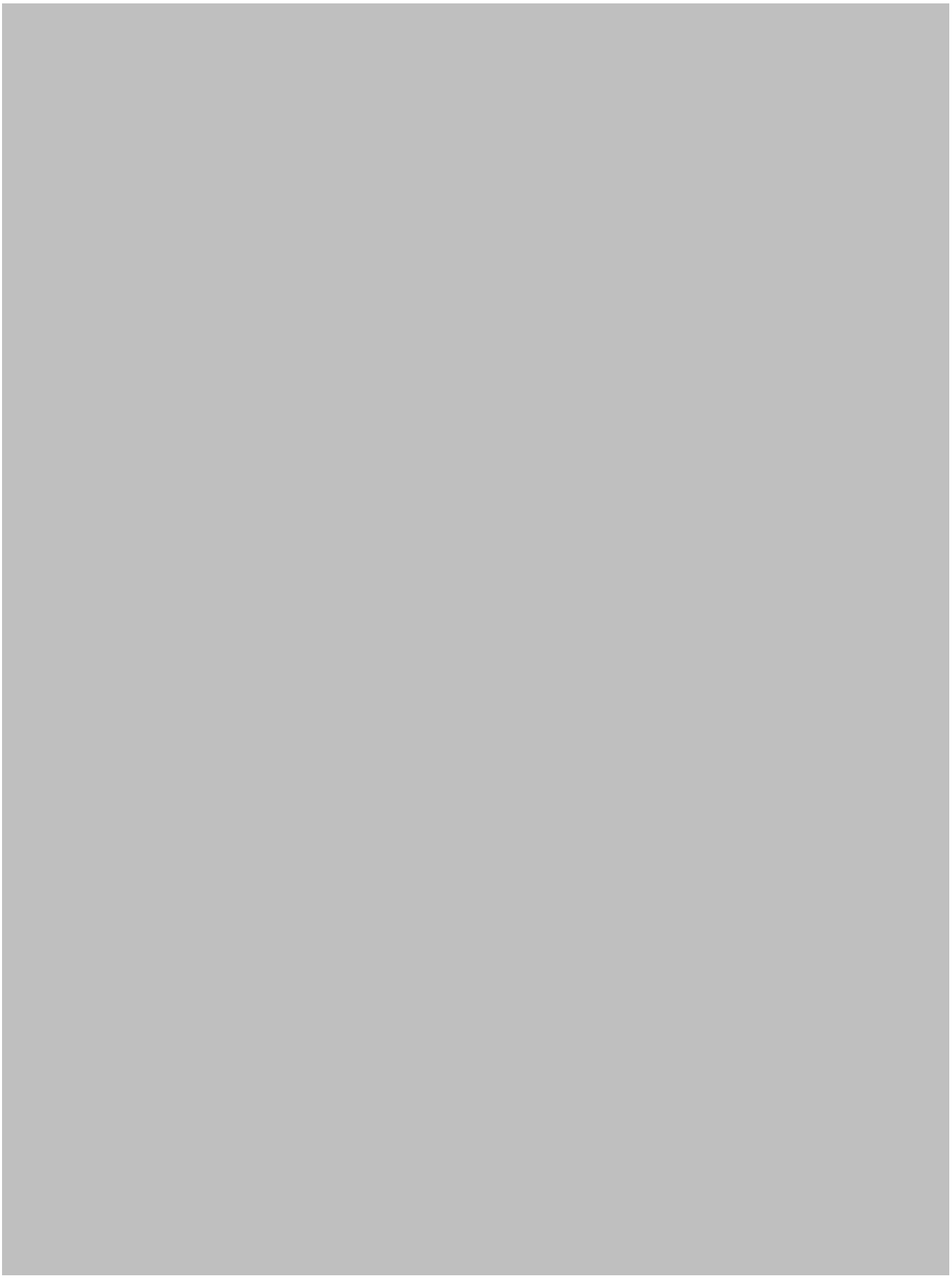


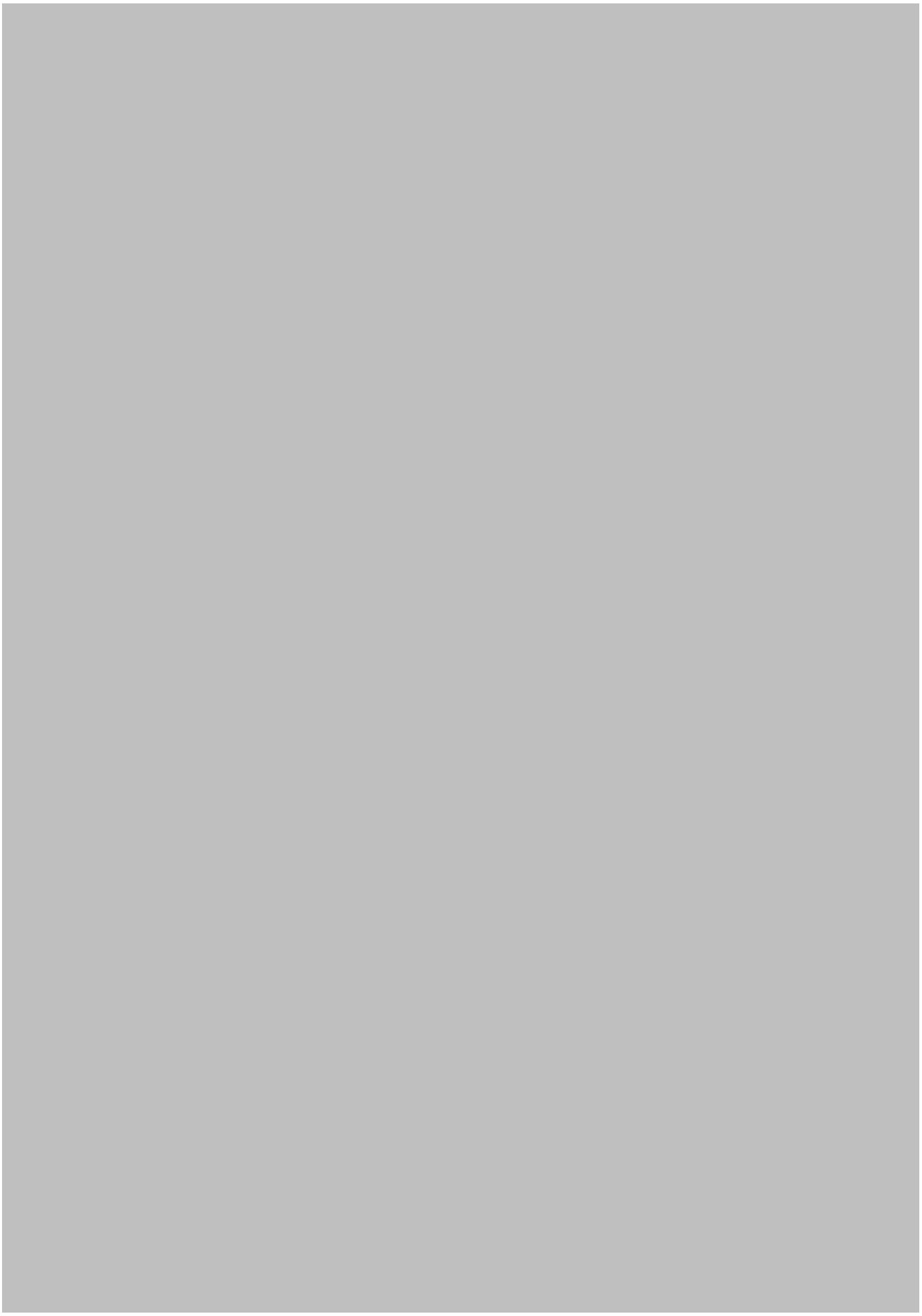












Appendix 5 Data Input Quality Control Standard Operating Procedure

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS Foundation Trust

Data Input Quality Control SOP: CANVAS

University of Birmingham

CANVAS

CMV Modulation of the Immune System in ANCA-Associated VASculitis

Data Input Quality Control Standard Operating Procedure

VERSION 1.0

25.06.2015

KEY CONTACT DETAILS

PRINCIPAL INVESTIGATOR
PROFESSOR LORRAINE HARPER

CO-INVESTIGATORS
DR MATTHEW MORGAN
PROFESSOR PAUL MOSS

RESEARCH FELLOW
DR DIMITRIOS CHANOUZAS

RESEARCH NURSES
ANNABEL GRINBERGS
LINDA COUGHLAN

THE PROCEDURES BELOW WILL BE PERFORMED EVERY TIME DATA IS USED FOR ANALYSIS (E.G. TO INFORM TRIAL STEERING COMMITTEE DECISIONS OR FOR PRELIMINARY / FINAL ANALYSIS OF THE DATA) AND THERE HAS BEEN A CHANGE IN DATA ENTRY FROM THE PREVIOUS TIME THAT QUALITY CONTROL (QC) WAS CARRIED OUT

PRIMARY OUTCOME

- DATA WILL BE TRANSCRIBED FROM SOURCE DATA TO AN SPSS FILE DIRECTLY FOR THE PURPOSES OF ANALYSIS
- DATA INTEGRITY WILL BE CHECKED VISUALLY ON THE SCREEN AND COMPARED TO THE SOURCE DATA PRINT-OUTS
- IN ADDITION DATA INTEGRITY WILL BE CHECKED BY PRINTING OUT THE SPSS DATA FILE AND DOUBLE CHECKING THE DATA ENTRIES ON THE SPSS FILE WITH THE DATA ENTRIES ON THE SOURCE DATA PRINT-OUTS
- FURTHERMORE AN ADDITIONAL PERSON WILL CHECK THE INTEGRITY OF THE DATA ENTRY ON THE SPSS FILE BY SELECTING 20% OF THE DATA AND CHECKING FOR ACCURACY OF TRANSCRIPTION BETWEEN THE SOURCE DATA PRINT-OUTS AND THE SPSS FILE
    - IF THE SELECTION OF DATA CHECKED IS CORRECT, DATA INPUT QUALITY CONTROL WILL BE COMPLETE
    - IF AN ERROR IS IDENTIFIED THEN A FURTHER 20% OF THE DATA WILL BE CHECKED AND THE DATA WILL BE CHECKED BY AN ADDITIONAL PERSON AS ABOVE
    - IF FURTHER ERRORS ARE IDENTIFIED THEN ALL DATA WILL BE CHECKED AND THE DATA WILL BE CHECKED AGAIN BY AN ADDITIONAL PERSON AS ABOVE
- DATA WILL ALSO BE TRANSCRIBED FROM SOURCE DATA TO THE CASE REPORT FORMS (CRF)
    - THE INTEGRITY OF THE DATA TRANSCRIPTION WILL BE CHECKED BY AN ADDITIONAL PERSON IN FULL

NOTE: VALUES OF <20 ON SOURCE DATA WILL BE TRANSCRIBED AS 0 ON THE SPSS FILE AS THE LIMIT OF DETECTION OF THE PCR ASSAY IS 20 COPIES. MISSING VALUES WILL BE INDICATED BY A VALUE OF 999999 ON THE SPSS FILE

SECONDARY OUTCOME

- DATA WILL BE TRANSCRIBED FROM SOURCE DATA (PDF FILE FLOW PLOTS) TO AN EXCEL SPREADSHEET
- DATA WILL THEN BE TRANSCRIBED FROM THE EXCEL SPREADSHEET TO AN SPSS FILE FOR THE PURPOSES OF ANALYSIS
- QUALITY CONTROL WILL TAKE PLACE AS ABOVE (PRIMARY OUTCOME) BY CHECKING ACCURACY OF TRANSCRIPTION FROM THE PDF FLOW PLOTS TO THE EXCEL SPREADSHEET TO THE SPSS FILE VISUALLY AND ON PRINT-OUTS

- An additional person will check the integrity of the data entries as above by selecting 20% of the data and checking for accuracy of transcription between the PDF flow plots to the excel spreadsheet to the SPSS file
- In the case that errors are identified the procedure detailed above (primary outcome) will be followed
- Data will also be transcribed from the excel spreadsheet to the CRF – This will be checked by an additional person by selecting 20% of the data for quality control as detailed above

The QC data sheets will be dated, signed and filed in the CANVAS Laboratory Folder

Appendix 6 CANVAS Laboratory Manual

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS Foundation Trust

LABORATORY MANUAL

University of Birmingham

CANVAS

CMV Modulation of the Immune System in ANCA-Associated VASculitis

Version 4.0

20.09.2015

Author:	**Dr Dimitrios Chanouzas**
Role:	**Analytical Project Manager**
Signature:	

TABLE OF CONTENTS

1. Contents Page 2
2. Key Names & Contact Details Page 3
3. Sample Pathway Page 4
4. Whole blood / positive control surface staining Work Instruction Page 6
5. Whole blood / positive control surface staining Assay Record Page 8
6. Processing of plasma by CRF Lab Page 9
7. Blood sample receipt and processing record Page 10
8. Processing of 6ml blood sample at month 1-5 and month 7-11 visits by CRF Lab Page 11
9. Processing of urine samples by CRF Lab Page 12

2. Key Names and Contact Details

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3. Sample Pathway

Following informed consent patient visits will take place at the NIHR / Wellcome Trust Clinical Research Facility (CRF) – see also CANVAS Sample Collection Work Instruction (APPENDIX A)

1. **5ml Blood** Sample Collection Prior to Baseline **(Pre-Baseline)**
    a. Collected in Green Lithium Heparin Tube
        i. Research Fellow to pick up from CRF
        ii. Sample used to determine CD3+CD4+CD28- % prior to randomisation
            1. Process sample according to Work Instruction detailed in Section 4 of this manual
            2. Keep assay record using form enclosed in Section 5 of this manual
            3. No sample storage necessary

2. **50ml Blood** Sample Collection **at baseline (0 months), 6 months and 12 months**
    a. **2** x 4ml EDTA (Purple) Tubes
        i. 1 x 4ml EDTA Tube sent to QE Virology Lab by CRF research nurse – Primary Outcome Determination (CMV PCR)
        ii. 1 x 4mL EDTA Tube given to CRF Lab by CRF research nurse
            1. Processed by CRF Lab according to agreed protocol detailed in Section 6 of this manual
                a. Sample will be used to determine plasma level of inflammatory mediators / cytokines – Secondary Outcome Determination
                b. Plasma samples will be stored at CRF freezers as per local procedures – sample inventory and freezer temperature logs kept locally
    b. **7** x 6ml Lithium Heparin (Green) Tubes
        i. Research Fellow to pick up from CRF
            1. Process sample and keep record according to Work Instruction detailed in Section 7 of this manual
                a. Plasma and PBMC pellet samples will be stored at Schools of Cancer Sciences and Immunity and Infection freezers – sample inventory kept within CANVAS Laboratory Folder; Freezer temperature logs kept locally by Schools of Cancer Sciences and Immunity and Infection
                b. PBMC samples will be stored at School of Immunity and Infection, Institute of Biomedical Research (IBR) liquid nitrogen tanks – sample inventory kept within CANVAS Laboratory Folder

2. Aliquot of whole blood used to determine CD3+CD4+CD28-% - Secondary Outcomes Determination (rest of sample to be used for exploratory research outcomes and stored as above)
    a. Process sample according to Work Instruction detailed in Section 4 of this manual
    b. Keep assay record using form enclosed in Section 5 of this manual

3. **10ml Blood** Sample Collection **at monthly visits (except 0, 6 and 12 month visits)**
    a. **1** x 4ml EDTA (Purple) Tube sent to QE Virology Lab by CRF research nurse – Primary Outcome Determination (CMV PCR)
    b. **1** x 6ml Lithium Heparin (Green) Tube given to CRF Lab by CRF research nurse
        i. Processed by CRF Lab according to agreed protocol detailed in Section 8 of this manual
            1. Sample used for exploratory research outcomes
            2. PBMC samples will be stored at School of Immunity and Infection, IBR liquid nitrogen tanks – sample inventory kept within CANVAS Laboratory Folder

4. **Urine** Sample Collection **at All Time Points (Except Pre-Baseline)**
    a. **5-10 ml** sent to QE Virology Lab by CRF research nurse – Primary Outcome Determination (CMV PCR)
    b. Rest of Urine Sample given to CRF Lab by CRF research nurse
        i. Sample processed according to CRF laboratory standard protocol outlined in Section 9 of this manual
            1. Sample used for exploratory research outcomes
            2. Urine samples will be stored at CRF freezers as per local procedures – sample inventory and freezer temperature logs kept locally

4. Whole blood / positive control surface staining Work Instruction

PURPOSE:

This Work Instruction describes the protocol for the surface staining of lymphocytes in whole blood / CD4 positive controls.

REAGENTS:

		Product No
▪ Cytofix CD4 Normal Positive Control	Cytomark / Caltag MedSystems	CF04-N
▪ Anti-Human CD3 Brilliant Violet 650	Biolegend	317323
▪ Anti-Human CD4 Brilliant Violet 605	Biolegend	317438
▪ Anti-Human CD28 eFluor 450	Ebioscience	48-0289-42
▪ Anti-Human CD4 eFluor 450	Ebioscience	48-0047-42
▪ Mouse IgG2a K Isotype Control BV 650	Biolegend	400265
▪ Mouse IgG2b K Isotype Control BV 605	Biolegend	400350
▪ Mouse IgG1 K Isotype Control eFluor450	Ebioscience	48-4714-82
▪ 1x RBC Lysis Buffer	Ebioscience	00-4333-57
▪ MACS Buffer	School of Cancer Sciences	

MATERIALS & METHODS:

- Disposable Gilson Pipette Tips
- 5 ml FACS tubes
- Pipette-Boy
- Full Set of Calibrated Pipettes
- Microbiological Safety Cabinet

WORK INSTRUCTION

1. Label 5ml FACS tubes.
2. Prepare master mix of CD3 (2uL / test), CD4 (2uL / test), and CD28 (3uL / test) antibodies on the day of intended use. Make up to total volume of 20uL per test with MACS buffer.
3. Aliquot 50uL of Cytofix CD4 Normal positive control or 100uL of whole blood into each tube.
4. Add the appropriate volume of CD3 CD4 CD28 master mix in each experimental tube and pulse vortex to mix.
5. *Also prepare 3 single stain compensation tubes and an unstained tube each containing 100uL of whole blood.*
6. *For each single stain compensation tube add the appropriate volume of CD3, CD4 or CD28 antibody (2uL / test for BV605 and BV650 and 3uL / test for eFluor450). Do not add antibody to the unstained tube.*
7. *Also prepare 1 Fluorescence Minus One (FMO) tube containing 100uL of whole blood.*
8. *For the FMO add the appropriate volume of antibody / isotype control as below.*

CD28 FMO
2uL BV 650 CD3
2uL BV 605 CD4
0.5uL eFluor 450 Isotype

9. Incubate for 30 minutes at 4 degrees Celsius in the dark.
10. Add 2ml of 1x RBC Lysis Buffer and pulse vortex.
11. Incubate for 15 minutes at room temperature in the dark.
12. Without washing centrifuge cells at 1600 rpm for 5 minutes, discard supernatant and gently resuspend cells.
13. Add 2ml MACS buffer per tube and centrifuge again as above.
14. Acquire the data on an LSR II Flow Cytometer and analyse the data using FACS DIVA Software.

5. Whole blood / positive control surface staining Assay Record

Whole Blood / Positive Control Surface Staining Assay Record

Date of Experiment _____

Type of Experiment (i.e. validation or clinical trial sample) _____

Unique Patient ID (if clinical trial sample) _____

Time point (i.e. 0, 6, 12 months if clinical trial sample) _____

Length of time from drawing blood sample to processing _____

Reagents used:

CD3 Brilliant Violet 650 Monoclonal Antibody	Lot_____	Expiry Date_____
CD4 Brilliant Violet 605 Monoclonal Antibody	Lot_____	Expiry Date_____
CD28 eFluor 450 Monoclonal Antibody	Lot_____	Expiry Date_____
CD4 eFluor 450 Monoclonal Antibody	Lot_____	Expiry Date_____
Brilliant Violet 650 Isotype Control	Lot_____	Expiry Date_____
Brilliant Violet 605 Isotype Control	Lot_____	Expiry Date_____
eFluor 450 Isotype Control	Lot_____	Expiry Date_____
Cytofix CD4 Normal Positive Control	Lot_____	Expiry Date_____
1x RBC Lysis Buffer Solution	Lot_____	Expiry Date_____

Pipettes used:

_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____

6. Processing of plasma by WTCRF Lab

Protocol for Processing of Plasma Samples – Author Dr Julie Williams (Former WTCRF Lab Manager) (edited by Dr D Chanouzas)

Receive	Processing	Storage
4ml **Purple EDTA** for plasma	Spin within 30min 10min 3000rpm at 4 degrees Celsius Divide between 4 tubes	Store at -80 degrees Celsius Samples will be stored at WTCRF facilities until the end of the study at which point they will be taken to University of Birmingham Laboratories for batch assaying / analysis.

7. Blood Sample Receipt and Processing Record – University of Birmingham – CANVAS Trial

Unique identifier _____

Date _____ *Time point in study* _____

Sample Received from WTCRF _____ *Sample Condition* _____

Length of time from drawing blood sample to processing _____

Received and Processed by _____

Remember to label all aliquots with ID, time-point, date, volume, type of material and concentration if applicable

FIRST USE SMALL ALIQUOT OF WHOLE BLOOD TO RUN SURFACE STAINING FOR 2° OUTCOME (Section 4, CANVAS Lab Manual)

Spin 1 green tube at 1600rpm for 10 minutes and aliquot plasma in 4 parts of 250uL
Location in -80 _____

Process blood to acquire PBMC
PBMC Count _____
PBMC used for fresh functional experiments _____

Process $1x10^6$ cells (x2) if enough sample for DNA
Spin in micro-centrifuge at 4000rpm for 5 minutes
Carefully aspirate supernatant and store at -80
Location in -80 _____

Freeze remainder of PBMC; Freeze as many cells as possible but ensure at least 2 tubes of $5x10^6$ cells each in 0.5ml are frozen to enable analysis of $1x10^6$ cells x 2.

Number of cells frozen _____
Number of cryovials, volume and concentration _____
Location in -80 _____ (Transfer to liquid nitrogen within 7 days)
Location in liquid nitrogen _____

8. Processing of 6ml blood sample at month 1-5 and month 7-11 visits by WTCRF Lab – Work Instruction

1. Centrifuge within 2 hours at 584 g for 10 minutes.
2. Carefully aspirate 250uL of plasma x 4 and aliquot in 4 tubes.
3. Label tubes with Sample Type, Sample Volume, Unique Patient ID, Patient Initials, Study Visit, Date and Study Name (CANVAS) and store at -80 degrees Celsius.
4. Invert green lithium heparin tube a few times to re-suspend blood sample again.
5. Dilute blood sample 1:1 with RPMI pre-warmed to 37 degrees Celsius: Pour contents of blood sample tube in a 50ml tube, then add 5ml RPMI into empty blood sample tube and mix with remaining blood. Pour RPMI from the blood sample tube into the 50ml tube containing the blood sample.
6. Add 15ml Ficoll Paque into a fresh 50ml tube.
7. Carefully layer the diluted blood sample over the Ficoll layer in order to get two distinct layers.
8. Spin at **room** temperature at 584 g for 30 minutes with the **brake off**.
9. Carefully harvest the buffy coat using a Pasteur pipette into a fresh 50ml tube.
10. Top up to 40ml with RPMI and spin at 912 g for 7 minutes.
11. Carefully discard supernatant and re-suspend the PBMC pellet.
12. Top up to 40ml with RPMI and spin at 584 g for 7 minutes.
13. Carefully discard supernatant and re-suspend the PBMC pellet.
14. Add 10ml of RPMI and take 10uL aliquot to count cells using a haemocytometer or automated cell counter.
15. Centrifuge sample at 584 g for 7 minutes.
16. Carefully discard supernatant and re-suspend the cells at 20 x 10^6 cells / ml in Freezing Solution A.
17. Add an equal volume of freezing solution B to the cells (dropwise fashion) with gentle agitation of the tube.
18. Transfer to cryovials – label cryovials with Sample type, Sample Volume, Unique Patient ID, Patient Initials, Study Visit, Date and Study Name (CANVAS).
19. Place cryovials in Mr Frosty for minimum of 4 hours at -80 degrees Celsius.
20. Transfer to liquid nitrogen within 7 days.

Freezing Solution A: 60% Fetal calf serum (FCS) and 40% RPMI

Freezing Solution B: 20% DMSO and 80% FCS

9. **Processing of urine samples by WTCRF Lab**

- **Mix well**
- **Split into two aliquots**
- **Label Aliquot A as Unspun along with the other agreed identifiers and store at -80 degrees Celsius**
- **Label Aliquot B as Spun along with the other agreed identifiers; Spin at 1000 rpm, at room temperature for 1 minute; Store at -80 degrees Celsius**
- **Samples will be stored at WTCRF facilities until the end of the study at which point they will be taken to University of Birmingham Laboratories for assaying / analysis.**

APPENDIX A

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS Foundation Trust

Sample Collection WORK INSTRUCTION: CANVAS

University of Birmingham

CANVAS

CMV Modulation of the Immune System in ANCA-Associated VASculitis

WORK INSTRUCTION for Sample Collection

VERSION 1.2

15.06.2015

13

Clinical Trial Participants (Intended number = 50)

Following informed consent patient visits will take place at the Wellcome Trust Clinical Research Facility (WTCRF)

5. **5ml Blood** Sample Collection Prior to Baseline **(Pre-Baseline)**
    a. Collect in Green Lithium Heparin Tube - Label with Unique Patient ID, Patient Initials, Study Visit, Date and Study Name (CANVAS)
        i. Contact Research Fellow (07971402309) to collect within 30 minutes of blood being drawn

6. **50ml Blood** Sample Collection **at baseline (0 months), 6 months and 12 months**
    a. **2** x 4ml EDTA (Purple) Tubes
        i. Send 1 x 4ml EDTA Tube to QE Virology – Ensure Fully Labelled and P386 Sticker Affixed
        ii. Send 1 x 4mL EDTA Tube to WTCRF Lab – Label with Unique Patient ID, Patient Initials, Study Visit, Date and Study Name (CANVAS)
            1. Spin down according to agreed protocol (Appendix A) and aliquot plasma to 4 parts (Label as above) – freeze at -80 degrees Celsius
    b. **7** x 6ml Lithium Heparin (Green) Tubes – Label with Unique Patient ID, Patient Initials, Study Visit, Date and Study Name (CANVAS)
        i. Contact Research Fellow (07971402309) to collect Green Tubes within 30 minutes of blood being drawn

7. **10ml Blood** Sample Collection **at monthly visits (except 0, 6 and 12 month visits)**
    a. **1** x 4ml EDTA (Purple) Tube
        i. Send to QE Virology – Ensure Fully Labelled and P386 Sticker Affixed
    b. **1** x 6ml Lithium Heparin (Green) Tube – Label with Unique Patient ID, Patient Initials, Study Visit, Date and Study Name (CANVAS)
        i. Send to WTCRF Lab
            1. Process according to agreed protocol (Appendix C)

8. **Urine** Sample Collection **at All Time Points (Except Pre-Baseline)**
    a. Send **5-10 ml** to QE Virology – Ensure Fully Labelled and P386 Sticker Affixed
    b. Rest of Urine Sample to be sent to WTCRF Lab – Label with Unique Patient ID, Patient Initials, Study Visit, Date and Study Name (CANVAS)
        i. Spin Down according to WTCRF laboratory standard protocol (Appendix B) and Aliquot into 2 parts (Label as Above)
            1. Freeze at -80 Degrees Celsius

Any problems or questions please contact Linda Coughlan, Annabel Grinbergs or Dr Dimitrios Chanouzas

APPENDIX A

Protocol for Processing of Plasma Samples – Author Dr Julie Williams (Former WTCRF Lab Manager) (edited by Dr D Chanouzas)

Receive	Processing	Storage
4ml **Purple EDTA** for plasma	Spin within 30min 10min 3000rpm at 4 degrees Celsius Divide between 4 tubes	Store at -80 degrees Celsius Samples will be stored at WTCRF facilities until the end of the study at which point they will be taken to University of Birmingham Laboratories for batch assaying / analysis.

APPENDIX B

Protocol for Processing of Urine Samples

- **Mix well**
- **Split into two aliquots**
- **Label Aliquot A as Unspun along with the other agreed identifiers and store at -80 degrees Celsius**
- **Label Aliquot B as Spun along with the other agreed identifiers; Spin at 1000 rpm, at room temperature for 1 minute; Store at -80 degrees Celsius**
- **Samples will be stored at WTCRF facilities until the end of the study at which point they will be taken to University of Birmingham Laboratories for assaying / analysis.**

APPENDIX B

Retrospective note on sample processing for exploratory analyses

1. Sample processed as per "Blood sample receipt and processing record" detailed in page 10 of this manual.
2. Following aliquoting of fresh whole blood for CD4+CD28- cell enumeration (secondary outcome) and aliquoting of plasma, the sample is used for harvesting peripheral blood mononuclear cells (PBMC).
3. The method for preparing PBMC is as detailed in page 18 of this manual.
4. Following processing, PBMC are used to set up stimulation cultures overnight (16 hours; 14-20 hours allowed) where PBMC are unstimulated, stimulated by staphylococcal enterotoxin B (SEB) or stimulated by CMV lysate (5×10^5 – 1×10^6 PBMC used per tube).
5. For each patient time point 4 stimulation tubes are set up (total volume 500uL):

U – unstimulated: monensin 1:1000, CD154 PE 5uL, PBMC, supplemented RPMI

SEB – SEB stimulated: monensin 1:1000, CD154 PE 5uL, SEB 10uL, supplemented RPMI

CMV Ly A – CMV Lysate stimulated panel A: monensin 1:1000, CMV lysate 5uL, supplemented RPMI

CMV Ly B – CMV Lysate stimulated panel B: monensin 1:1000, CMV lusate 5uL, CD154 PE 5uL, supplemented RPMI

6. Following incubation at 37 degrees Celsius 5% CO2 the cells are washed twice in PBS, then stained with viability dye for 30 minutes at 4 degrees Celsius (eFluor 506 1:10, Ebioscience).
7. Cells are then washed with PBS once and MACS buffer once and stained with the surface antibodies (see below) for 30 minutes at 4 degrees Celsius.
8. Following a wash in MACS buffer, cells are vortexed to completely dissociate the pellet and fixed and permeabilised using the Ebioscience FoxP3 kit as per manufacturer's instructions.
9. Immediately following this, cells are stained with intracellular antibodies (see below) at 4 degrees for 30 minutes, followed by a final wash in MACS buffer before acquiring events in an LSR II Flow Cytometer using DIVA software.

Panels

U / SEB: FITC T-bet, PE CD-154, PE-CF594 IFN-g, PerCP eFluor 710 IL-2, PE-Cy7 IL-10, eFluor450 CD28, eFluor 506 Viability, BV605 CD4, BV 650 CD3, APC EOMES, AF700 TNF-a

A: FITC T-bet, PE NKG2D, PE-CF594 IFN-g, PerCP eFluor 710 IL-2, PE-Cy7 IL-10, eFluor 450 CD28, eFluor 506 Viability, BV605 CD4, BV650 CD3, APC EOMES, AF700 TNF-a

B: FITC LAG-3, PE CD-154, PE-CF594 IFN-g, PE-Cy5 CTLA-4, PerCP eFluor 710 IL-2, PE-Cy7 TIM-3, eFluor 450 CD28, eFluor 506 Viability, BV605 CD4, BV650 CD3, APC BLIMP-1, AF700 TNF-a, APC-Cy7 PD-1

Appendix 7 Validation Plans and Analysis for Clinical Trial Laboratory Assays

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS Foundation Trust

ASSAY VALIDATION ANALYSIS: CANVAS

University of Birmingham

CANVAS

CMV Modulation of the Immune System in ANCA-Associated VASculitis

Surface Staining of CD3 CD4 CD28 in Whole Blood (PART 1)

Version 1.0

Author:	**Dr Dimitrios Chanouzas**
Role:	**Analytical Project Manager**
Signature:	

	Signature	**Name (Print)**	**Date**
Approved By:			
Authorised By:			

TABLE OF CONTENTS

1. Contents Page 2
2. Key Names & Contact Details Page 3
3. Assay Validated Page 4
4. Assay Methodology Page 4
5. Validation Parameters Page 4
6. Characterisation of data analysis Page 5
7. Study location and duration Page 5
8. Location of study records and primary data Page 5
9. Assay Validation Analysis Report Page 6
10. Acceptance Criteria Page 7
11. Appendix 1. Whole blood surface staining work instruction Page 8
12. Appendix 2. References Page 9
13. Appendix 3. Primary FACS data Page 10

3. ASSAY VALIDATED

3.1 Objective

The aim of this validation study is to establish that the performance characteristics of the assay that will be used to surface stain lymphocytes in whole blood for CD3, CD4 and CD28, meets the analytical requirements of the CANVAS trial.

3.2 Justification

Cytomegalovirus (CMV) is a herpes virus that infects the majority of the UK population aged ≥ 65, and like other herpes viruses is never cleared from the host. The cellular immune response directed against CMV is critical in preventing viral reactivation but also results in an accumulation of virus specific T cells. It has previously been shown that an accumulation of CD4+CD28- T-cells is associated with an increased risk of infection as well as an increased risk of mortality amongst patients with ANCA-associated vasculitis (AAV) [1].

CANVAS will randomise 50 patients with stable AAV to receive treatment with an antiviral drug (valaciclovir) or not, over a 6 month period. One of the pre-specified secondary outcomes is whether treatment with valaciclovir induces a change in the proportion of CD4+ CMV-specific T-cells previously identified as CD4+ T-cells that have lost expression of the co-stimulatory molecule CD28[1].

Surface staining of lymphocytes within whole blood followed by red blood cell lysis is a well documented technique that will be used to determine the change in proportion of CD3+CD4+CD28- T-cells from baseline to 6 months.

3.3 Whole Blood CD3 CD4 CD28 Staining: Overview

- Whole blood is collected and a 1ml aliquot is set aside for whole blood surface staining.
- An aliquot of whole blood (100uL) is transferred to a FACS tube and stained directly with a panel of antibodies to allow detection of the surface markers CD3, CD4 and CD28.
- The whole blood is then incubated with the antibodies at 4°C for 30 minutes in the dark.
- Stained whole blood is then treated with E-bioscience 1x Red Blood Cell Lysis Buffer in order to lyse red blood cells and incubated for 15 minutes in the dark at room temperature.
- Following 2 washes in MACS buffer the cells can then be acquired using a flow cytometer and the data analysed using flow cytometric specific software.

4. ASSAY METHODOLOGY

The assay was conducted in accordance with the work instruction specified in the appendix.

5. Validation Parameters

The whole blood surface staining assay permits the frequency of CD3+CD4+CD28- lymphocytes to be measured.

The following parameters have been validated:

1. The period of time blood can be left between isolation from the patient and staining
2. Precision and Reproducibility

5.2 Period of time blood can be left between isolation from the patient and staining:

A critical component of the CANVAS trial is determining if the use of valaciclovir results in any alteration in the $CD4^{+}CD28^{-}$ cell populations in whole blood. To ensure that any change we observe in cell populations is a direct effect of the drug and not a consequence of deterioration of patient blood samples, samples from healthy CMV positive donors were isolated in the morning and left at room temperature for 1 hour, 3 hours and 5 hours before being analysed. Previous validation studies within the University of Birmingham have identified that blood samples are to be kept at room temperature as opposed to storing them at 4°C. The outcome of this validation study will ensure that blood samples collected are processed in an appropriate time period that does not result in deterioration of surface marker staining.

5.3 Precision & Reproducibility:

Inter-assay variation

Due to the nature of the CD4+CD28- frequency being altered by the level of subclinical CMV reactivation, this parameter was not validated. The degree of variation in the CD4+CD28- frequency caused by the level of CMV reactivation will be a focus of a research aspect of this broader study.

Intra-assay variation

Samples were tested in duplicate to provide an indication of reproducibility and to determine whether the coefficient of variation is acceptable to enable running single samples for the purposes of the clinical trial.

6. Characterisation of data analysis

Every whole blood surface staining assay was acquired using the LSRII flow cytometer and FACS Diva software. The resulting data were retrospectively analysed using FACS Diva software.

7. Study location and duration

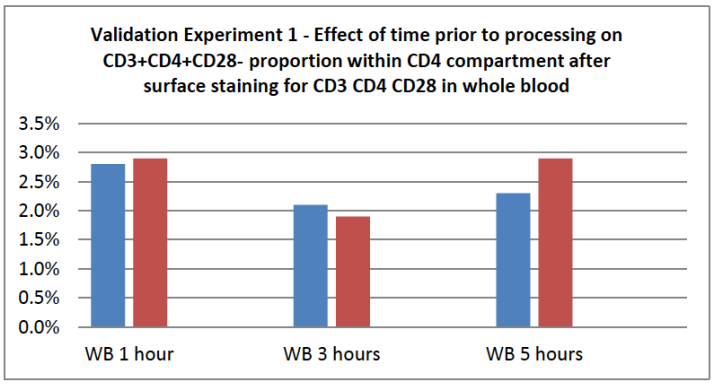
This validation study was undertaken at the Clinical Trials Laboratory (Room 416), School of Cancer Sciences, University of Birmingham, between May and July 2013. The Test Operator and Analytical Project Manager was Dr Dimitrios Chanouzas.

8. Location of study records and primary data

All relevant details and data were recorded into a bound laboratory notebook by the Test Operator. Primary electronic a following analysis of FACS data are stored in the University of Birmingham U Drive of the Test Operator whilst paper copies are filed in the CANVAS FACS Folder and attached as an appendix to this report.

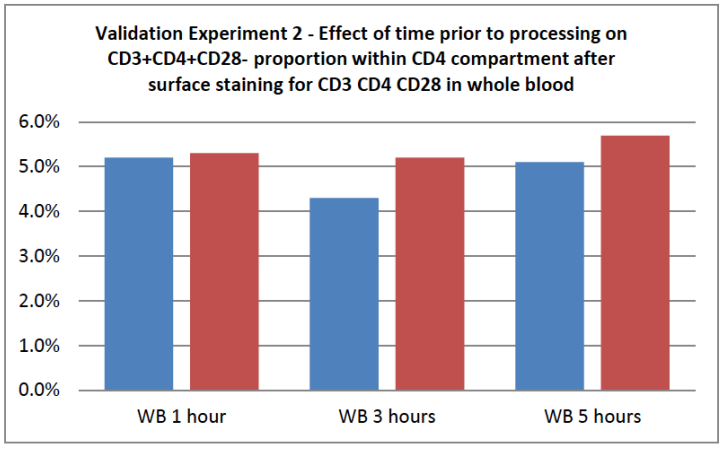
9. Assay Validation Analysis Report

In order to examine whether the period of time blood is left at room temperature prior to processing affects the proportion of CD3+CD4+CD28- cells within the CD4 compartment, blood was drawn from healthy volunteers and left at room temperature for 1 hour, 3 hours and 5 hours before being analysed as described in the appendix. This experiment was repeated three times. The one-sample Wilcoxon signed rank test was used to determine whether the median CD3+CD4+CD28- proportion at 3 hours and 5 hours was statistically different to that at 1 hour. In order to test intra-assay variability all experiments were carried out in duplicate and the coefficient of variation was calculated.



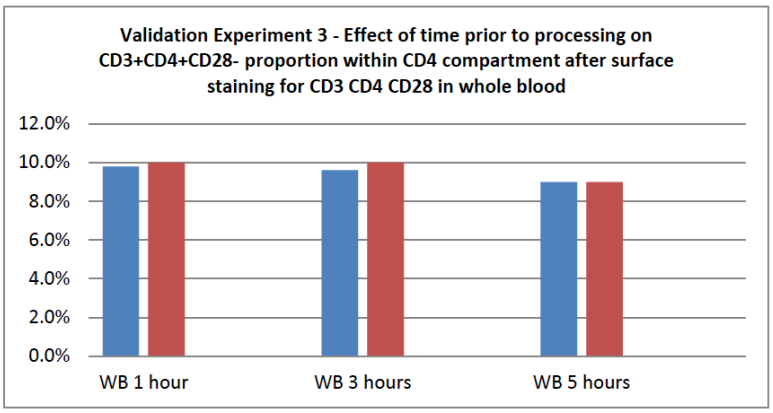
Coefficient of variation: 2.5% (1 hour), 7.1% (3 hour), 16.3% (5 hour)

Wilcoxon signed rank test (hypothesised median 2.85%): p value 0.18 (3 hour), 0.66 (5 hour)



Coefficient of variation: 1.3% (1 hour), 13.4% (3 hour), 7.9% (5 hour)

Wilcoxon signed rank test (hypothesised median 5.25%): p value 0.18 (3 hour), 0.66 (5 hour)



Coefficient of variation: 1.4% (1 hour), 2.9% (3 hour), 0% (5 hour)

Wilcoxon signed rank test (hypothesised median 5.25%): p value 0.66 (3 hour), 0.16 (5 hour)

10. Acceptance Criteria

There was no statistically significant difference between the mean CD3+CD4+CD28- % at 3 hours and 5 hours versus 1 hour at all experiments. Therefore it was decided that following collection, blood samples can be left at room temperature for up to a maximum of 5 hours before processing. However, in order to minimise variation patient appointments will be scheduled so that blood sample processing can occur within 1-3 hours of drawing the sample.

The coefficient of variation was acceptable throughout the validation analysis study. However it was decided that a positive control with a pre-defined accepted range of CD3+CD4+CD28+ cells was necessary in order to properly validate the various components of the assay in terms of intra-assay and inter-assay variability and also allow running of an aliquot of the positive control with each experimental run.

11. Appendix 1:

Whole Blood Surface Staining Work Instruction

PURPOSE:

This Work Instruction describes the protocol for the surface staining of lymphocytes in whole blood.

REAGENTS:

▪ Whole blood	Patient or Control Donor
▪ Anti-Human CD3 Brilliant Violet 650	Biolegend
▪ Anti-Human CD4 Brilliant Violet 605	Biolegend
▪ Anti-Human CD28 eFluor 450	Ebioscience
▪ 1x Red Blood Cell Lysis Buffer	Ebioscience
▪ MACS Buffer	Cancer Sciences

MATERIALS & METHODS:

- Disposable Gilson Pipette Tips
- 5 ml FACS tubes
- Pipette-Boy
- Full Set of Calibrated Pipettes
- Centrifuge
- Microbiological Safety Cabinet

WORK INSTRUCTION

1. Label 5ml FACS tubes.
2. Prepare master mix of CD3 (3uL / test), CD4 (3uL / test), and CD28 (3uL / test) antibodies on the day of intended use.
3. Aliquot 100uL of whole blood into each tube.
4. Add the appropriate volume of CD3 CD4 CD28 master mix in each experimental tube and pulse vortex to mix.
5. Incubate for 30 minutes at 4 degrees Celsius in the dark.
6. Add 2ml of 1x Red Blood Cell Lysis Buffer, pipette up and down to mix and pulse vortex.
7. Incubate for 15 minutes at room temperature in the dark.
8. Centrifuge at 1600 rpm for 5 minutes at room temperature.
9. Discard the supernatant and gently resuspend the pellet by agitating the tube running the bottom of the tube along the rack.
10. Add 2ml of MACS buffer and centrifuge at 1600 rpm for 5 minutes at room temperature.
11. Discard the supernatant and gently resuspend the pellet as above (step 9).
12. Repeat the wash described in step 10 and resuspend the pellet.
13. Acquire the data on an LSR II Flow Cytometer and analyse the data using FACS DIVA Software.

12. Appendix 2:

REFERENCES

1. Morgan MD, Pachnio A, Begum J, *et al*. CD4+CD28- T cell expansion in granulomatosis with polyangiitis (Wegener's) is driven by latent cytomegalovirus infection and is associated with an increased risk of infection and mortality. Arthritis Rheum 2011;63(7):2127-37.

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS Foundation Trust

ASSAY VALIDATION ANALYSIS: CANVAS

University of Birmingham

CANVAS

CMV Modulation of the Immune System in ANCA-Associated VASculitis

Surface Staining of CD3 CD4 CD28 in Whole Blood

(PART 2 -Incorporating Positive Controls)

Version 1.0

Author:	**Dr Dimitrios Chanouzas**
Role:	**Analytical Project Manager**
Signature:	

	Signature	**Name (Print)**	**Date**
Approved By:			
Authorised By:			

TABLE OF CONTENTS

1. Contents Page 2
2. Key Names & Contact Details Page 3
3. Assay Requiring Validation Page 4
4. Assay Methodology Page 4
5. Validation Parameters Page 4
6. Characterisation of data analysis Page 8
7. Acceptance Criteria Page 8
8. Appendix 1. Whole blood / Positive Control surface staining work instruction Page 9
9. Appendix 2. Whole blood / Positive Control surface staining assay record Page 11
10. Appendix 3. References Page 12
11. Primary FACS Data Page 13

3. ASSAY REQUIRING VALIDATION

3.1 Objective

The aim of this validation study is to establish that the performance characteristics of the assay that will be used to surface stain lymphocytes in whole blood for CD3, CD4 and CD28, meets the analytical requirements of the CANVAS trial.

3.2 Justification

Cytomegalovirus (CMV) is a herpes virus that infects the majority of the UK population aged ≥ 65, and like other herpes viruses is never cleared from the host. The cellular immune response directed against CMV is critical in preventing viral reactivation but also results in an accumulation of virus specific T cells. It has previously been shown that an accumulation of CD4+CD28- T-cells is associated with an increased risk of infection as well as an increased risk of mortality amongst patients with ANCA-associated vasculitis (AAV) [1].

CANVAS will randomise 50 patients with stable AAV to receive treatment with an antiviral drug (valaciclovir) or not, over a 6 month period. One of the pre-specified secondary outcomes is whether treatment with valaciclovir induces a change in the proportion of CD4+ CMV-specific T-cells previously identified as CD4+ T-cells that have lost expression of the co-stimulatory molecule CD28[1].

Surface staining of lymphocytes within whole blood followed by red blood cell lysis is a well-documented technique that will be used to determine the change in proportion of CD3+CD4+CD28- T-cells from baseline to 6 months.

3.3 Whole Blood CD3 CD4 CD28 Staining incorporating positive controls: Overview

- Whole blood is collected and a 1ml aliquot is set aside for whole blood surface staining.
- An aliquot of whole blood or Cytofix CD4 Positive Control (50uL) is transferred to a FACS tube and stained directly with a panel of antibodies to allow detection of the surface markers CD3, CD4 and CD28.
- The whole blood / positive control is then incubated with the antibodies at 4°C for 30 minutes in the dark.
- Stained whole blood / positive control is then treated with EBioscience 1x RBC Lysis buffer in order to lyse red blood cells and incubated for 15 minutes in the dark at room temperature.
- Following washing in MACS buffer, cells are acquired using a flow cytometer (LSRII) and the data analysed using flow cytometric specific software (FACS DICA).

4. ASSAY METHODOLOGY

The assay will be conducted in accordance with the work instruction specified in the appendix.

5. Validation Parameters

The whole blood surface staining assay permits the frequency of CD3+CD4+CD28- lymphocytes to be measured.

The following parameters have been planned to be validated:

1. The period of time blood can be left between isolation from the patient and staining
2. Precision and Reproducibility

5.2 Period of time blood can be left between isolation from the patient and staining:

This aspect has been previously validated (Surface Staining of CD3 CD4 CD28 in Whole Blood (PART 1); Assay Validation Analysis). There was no statistically significant difference between the mean CD3+CD4+CD28- % at 3 hours and 5 hours versus 1 hour at all experiments. Therefore it was decided that following collection, blood samples can be left at room temperature for up to a maximum of 5 hours before processing. However, in order to minimise variation, patient appointments will be scheduled so that blood sample processing can occur within 1-3 hours of drawing the sample.

5.3 Precision & Reproducibility:

The precision and reproducibility of the whole blood staining assay will be validated using commercially available positive controls (Cytofix CD4 Normal Range Positive Control) manufactured by Cytomark / Caltag MedSystems. Cytofix CD4 controls are a stabilised preparation of whole human blood for use as a positive control when evaluating CD4 enumeration techniques. They provide verification of monoclonal antibody reagents and red blood cell lysis as well as validation of instrument performance and operator technique. Each lot of Cytofix CD4 positive control is provided with a quality control statement inclusive of the expected range of CD3+CD4+ lymphocytes within each lot.

Positive controls from the first lot will be initially assayed in triplicate on five separate experiments in order to define inter-assay and intra-assay variability as well as define acceptance criteria for the assay based on the CD3+CD4+CD28+ range. If the coefficient of variation in terms of intra-assay variability is acceptable this will enable experimental samples to be run singly for the purposes of the clinical trial.

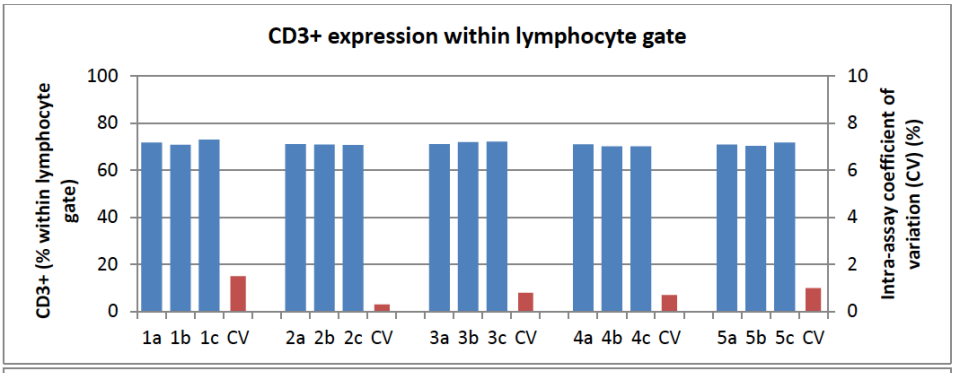
Fluorescence minus one (FMO) controls will be run for each of these experiments in order to guide gating strategies.

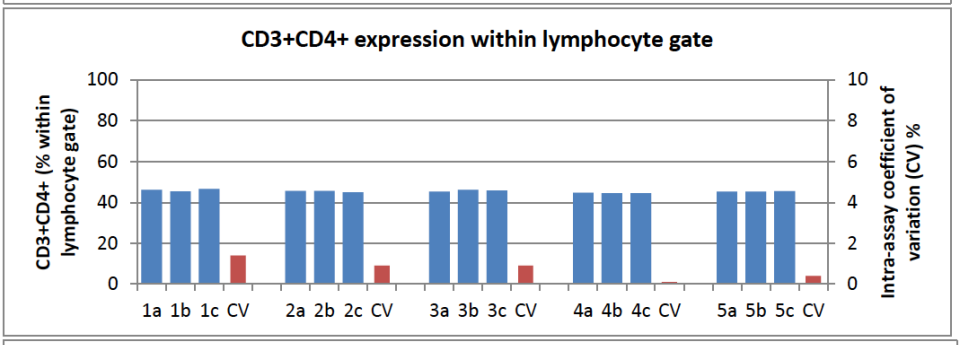
It is envisaged that when running experimental samples for the clinical trial, an aliquot of the Cytofix CD4 positive control will be run alongside each experiment in order to ensure that the CD3+CD4+CD28+ frequency in the positive control falls within the pre-defined acceptance criteria. FMO controls may be run for the CD28 fluorochrome depending on the initial results, however it is not expected that FMO controls for CD3 and CD4 will be required routinely.

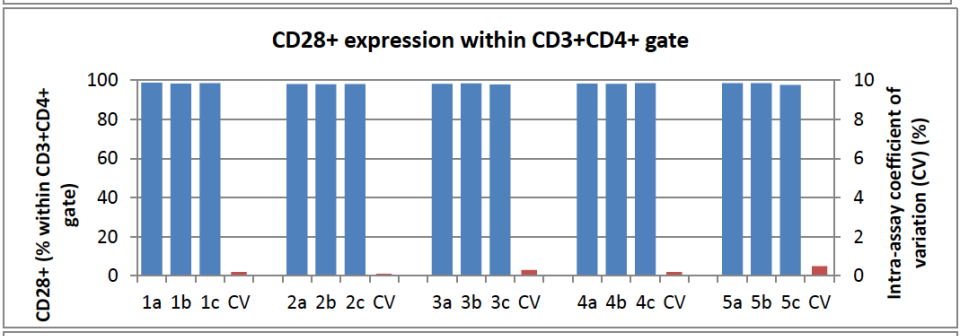
The expected CD3+CD4+CD28+ range for the purposes of acceptance criteria for each new lot of Cytofix CD4 controls will be re-validated by running a sample from the new lot in triplicate against a single aliquot from the previous lot.

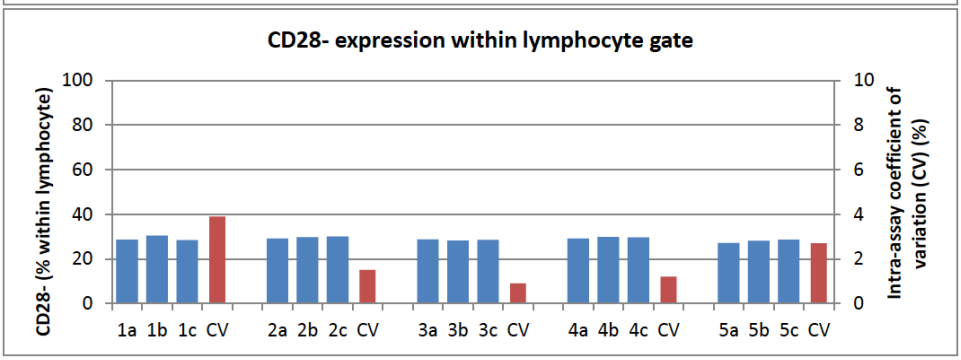
RESULTS

Intra-Assay Variability









Inter-Assay Variability

Table 1

	Coefficient of variation (%)	Mean %	Accepted Range (+/- 2 standard deviations)
CD3+ within lymphocyte gate	0.9	71.2	70.0 – 72.4
CD3+CD4+ within lymphocyte gate	1.2	45.5	44.4 – 46.6
CD28+ within CD3+CD4+ gate	0.2	98.2	97.7 – 98.7
CD28- within lymphocyte gate	2.5	28.9	27.5 – 30.4

Validation of length of time following processing that samples can be left at 4 degrees Celsius without change in signal prior to acquiring on LSR Flow Cytometer

Table 2

	4 hours – Exp. 1	4 hours – Exp. 1	4 hours – Exp. 2	4 hours – Exp. 2	10 hours	10 hours
	Mean % of 3 runs	Intra-assay Coefficient of variation (%)	Mean % of 3 runs	Intra-assay Coefficient of variation (%)	Mean % of 3 runs	Intra-assay Coefficient of variation (%)
CD3+ within lymphocyte gate	71.3	0.8	71.2	0.5	72.2	0.9
CD3+CD4+ within lymphocyte gate	45.7	1.2	45.6	1.2	46.2	1.5
CD28+ within CD3+CD4+ gate	98.6	0.3	98.7	0.2	98.4	0.1
CD28- within lymphocyte gate	29.5	2.5	29.3	0.9	28.0	1.8

All values fall within the accepted ranges defined in Table 1.

6. Characterisation of data analysis

Every whole blood surface staining assay was acquired using the LSRII flow cytometer and FACS Diva software. The resulting data was retrospectively analysed using FACS Diva software.

7. Acceptance Criteria

- Accepted ranges for the Cytofix CD4 positive control were defined for the relevant cell populations as detailed in Table 1.
- As the intra-assay and inter-assay coefficients of variation were excellent it was decided that both experimental samples and positive controls can be run singly for each experiment.
- An aliquot of the Cytofix CD4 positive control will be run alongside each experimental run to ensure that the relevant cell populations within the positive control fall within the above accepted ranges therefore providing on-going quality assurance of the precision of this assay.
- The accepted ranges of the relevant cell populations will be re-validated for each new lot of Cytofix CD4 positive control by running a sample from the new lot in triplicate against a single aliquot from the previous lot.
- FMO controls for the CD3 BV650 and CD4 BV605 monoclonal antibodies will not be routinely run with the exception of when a new lot of CD3 BV650 or CD4 BV605 monoclonal antibody is used.
- A single FMO control for the CD28 eFluor450 monoclonal will be run alongside each experiment as a gating aid to begin with. After the first 10 runs this will be re-evaluated to determine whether an FMO control needs to be run routinely for each experiment.
- Following processing samples will be analysed on the LSR Flow Cytometer within 5 hours.

8. Appendix 1:

Whole Blood / Positive Control Surface Staining Work Instruction

PURPOSE:

This Work Instruction describes the protocol for the surface staining of lymphocytes in whole blood / CD4 positive controls.

REAGENTS:

		Product No
▪ Cytofix CD4 Normal Positive Control	Cytomark / Caltag MedSystems	CF04-N
▪ Anti-Human CD3 Brilliant Violet 650	Biolegend	317323
▪ Anti-Human CD4 Brilliant Violet 605	Biolegend	317438
▪ Anti-Human CD28 eFluor 450	Ebioscience	48-0289-42
▪ Anti-Human CD4 eFluor 450	Ebioscience	48-0047-42
▪ Mouse IgG2a K Isotype Control BV 650	Biolegend	400265
▪ Mouse IgG2b K Isotype Control BV 605	Biolegend	400350
▪ Mouse IgG1 K Isotype Control eFluor450	Ebioscience	48-4714-82
▪ 1x RBC Lysis Buffer	Ebioscience	00-4333-57
▪ MACS Buffer	School of Cancer Sciences	

MATERIALS & METHODS:

- Disposable Gilson Pipette Tips
- 5 ml FACS tubes
- Pipette-Boy
- Full Set of Calibrated Pipettes
- Microbiological Safety Cabinet

WORK INSTRUCTION

1. Label 5ml FACS tubes.
2. Prepare master mix of CD3 (2uL / test), CD4 (2uL / test), and CD28 (3uL / test) antibodies on the day of intended use. Make up to total volume of 20uL per test with MACS buffer.
3. Aliquot 50uL of Cytofix CD4 Normal positive control or 100uL of whole blood into each tube.
4. Add the appropriate volume of CD3 CD4 CD28 master mix in each experimental tube and pulse vortex to mix.
5. *Also prepare 3 single stain compensation tubes and an unstained tube each containing 100uL of whole blood.*
6. *For each single stain compensation tube add the appropriate volume of CD3, CD4 or CD28 antibody (2uL / test for BV605 and BV650 and 3uL / test for eFluor450). Do not add antibody to the unstained tube.*
7. *Also prepare 3 Fluorescence Minus One (FMO) tubes each containing 100uL of whole blood.*
8. *For each FMO add the appropriate volume of antibody / isotype controls as below.*

CD3 FMO	CD4 FMO	CD28 FMO
0.26uL BV 650 Isotype	2uL BV 650 CD3	2uL BV 650 CD3
2uL BV 605 CD4	2uL BV 605 Isotype	2uL BV 605 CD4
3uL eFluor 450 CD28	3uL eFluor 450 CD28	0.5uL eFluor 450 Isotype

9. Incubate for 30 minutes at 4 degrees Celsius in the dark.
10. Add 2ml of 1x RBC Lysis Buffer and pulse vortex.
11. Incubate for 15 minutes at room temperature in the dark.
12. Without washing centrifuge cells at 1600 rpm for 5 minutes, discard supernatant and gently resuspend cells.
13. Add 2ml MACS buffer per tube and centrifuge again as above.
14. Acquire the data on an LSR II Flow Cytometer and analyse the data using FACS DIVA Software.

9. Appendix 2:

Whole Blood / Positive Control Surface Staining Assay Record

Date of Experiment _____

Type of Experiment (i.e. validation or clinical trial sample) _____

Unique Patient ID (if clinical trial sample) _____

Time point (i.e. 0, 6, 12 months if clinical trial sample) _____

Length of time from drawing blood sample to processing _____

Reagents used:

CD3 Brilliant Violet 650 Monoclonal Antibody	Lot_____	Expiry Date_____
CD4 Brilliant Violet 605 Monoclonal Antibody	Lot_____	Expiry Date_____
CD28 eFluor 450 Monoclonal Antibody	Lot_____	Expiry Date_____
CD4 eFluor 450 Monoclonal Antibody	Lot_____	Expiry Date_____
Brilliant Violet 650 Isotype Control	Lot_____	Expiry Date_____
Brilliant Violet 605 Isotype Control	Lot_____	Expiry Date_____
eFluor 450 Isotype Control	Lot_____	Expiry Date_____
Cytofix CD4 Normal Positive Control	Lot_____	Expiry Date_____
1x RBC Lysis Buffer Solution	Lot_____	Expiry Date_____

Pipettes used:

_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____

13. Primary FACS Data:

All primary FACS Data are available in the CANVAS Flow Analysis electronic folder.

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ASSAY VALIDATION PLAN: CANVAS

University of Birmingham

CANVAS

CMV Modulation of the Immune System in ANCA-Associated VASculitis

Luminex Assay

Version 1.0

Author:	**Dr Dimitrios Chanouzas**
Role:	**Analytical Project Manager**
Signature:	

	Signature	**Name (Print)**	**Date**
Approved By:			
Authorised By:			

TABLE OF CONTENTS

1. Contents Page 2
2. Key Names & Contact Details Page 3
3. Assay Requiring Validation Page 4
4. Assay Methodology Page 4
5. Validation Parameters Page 5
6. Characterisation of data analysis Page 5
7. Appendix 1. Work Instruction for Luminex Assay Page 6
8. Appendix 2. Luminex Assay Record Page 9
9. Appendix 3. Manufacturer CV values for intra and inter-assay variability Page 10
10. Appendix 4. References Page 11

3. ASSAY REQUIRING VALIDATION

3.1 Objective

The aim of this validation study is to establish that the performance characteristics of the assay that will be used to determine the plasma concentration of IL-2, TNF-α, IFN-γ, IL-6, IL-10, IL-17 and highly sensitive C-reactive protein (hsCRP) at baseline and at 6 months (secondary outcome), meets the analytical requirements of the CANVAS trial.

3.2 Justification

Cytomegalovirus (CMV) is a herpes virus that infects the majority of the UK population aged ≥ 65, and like other herpes viruses is never cleared from the host. The cellular immune response directed against CMV is critical in preventing viral reactivation but also results in an accumulation of virus specific T cells. It has previously been shown that an accumulation of CD4+CD28- T-cells is associated with an increased risk of infection as well as an increased risk of mortality amongst patients with ANCA-associated vasculitis (AAV) [1].

CANVAS will randomise 50 patients with stable AAV to receive treatment with an antiviral drug (valaciclovir) or not, over a 6 month period. One of the pre-specified secondary outcomes is whether treatment with valaciclovir induces a change in the concentration of soluble cytokines and markers of inflammation IL-2, TNF-a, IFN-g, IL-6, IL-10, IL-17 and hsCRP.

Determination of soluble cytokines and markers of inflammation in plasma by luminex is a well-documented technique that will be used to determine the change in concentration of the above stated cytokines and inflammatory markers from baseline to 6 months.

3.3 Luminex assay for the determination of the concentration of soluble cytokines and marker of inflammation in plasma: Overview

- Blood will be collected in EDTA blood tubes at baseline and at 6 months as described in the CANVAS Protocol and CANVAS Laboratory Manual.
- Blood will be processed to separate and collect the plasma that will be aliquoted and stored at -80 degrees Celsius as described in the CANVAS Laboratory Manual.
- The analyte-specific capture beads coated with target-specific capture antibodies will be added to the prepared sample. For determination of hsCRP, the manufacturer recommends dilution of plasma samples to 1:500 – therefore this will be run separately. For determination of all other markers, sample will be used neat and the analyte specific capture beads will be plexed together.
- Following a 2 hour incubation and wash step, the captured bead / analytes will be incubated with biotinylated analyte-specific detection antibody for 30 minutes, followed by a wash step.
- In order to quantitate the analyte, a fluorescent detection label will be added and allowed to incubate for 30 minutes, followed by a wash step.
- Samples will be read using a Biorad Luminex 200 instrument (Centre for Translational Inflammation Research, CTIR). The instrument is maintained at GLP standards with monthly calibration, daily validation on days that the instrument is being used, documentation of use and completion of appropriate cleaning procedures and documentation of structured training prior to an individual using the instrument.
- Results will be analysed with the ProcartaPlex Analyst Software 1.0 (Ebioscience).

4. ASSAY METHODOLOGY

The assay will be conducted in accordance with the work instruction specified Appendix 1.

5. Validation Parameters

The Luminex assay permits the plasma concentration of IL-2, TNF-α, IFN-γ, IL-6, IL-10, IL-17 and hsCRP to be measured in a multiplex immunoassay.

This validation plan will discuss the following parameters:

1. Precision and Reproducibility

5.1 Precision & Reproducibility:

The Luminex multiplex immunoassay has excellent precision and reproducibility as evidenced by low coefficient of variation (CV) values with regards to intra-assay and inter-assay variability (See Appendix 3 for manufacturer CV values). This is a commercial assay that has been rigorously validated by the company. Hence no further validation is planned.

6 Characterisation of data analysis

Data from the luminex assay will be acquired using a Biorad Luminex 200 instrument. Analysis will be carried out retrospectively using the ProcartaPlex Analyst Software 1.0.

7 Appendix 1:

Luminex Assay Work Instruction

PURPOSE:

This Work Instruction describes the protocol for determination of cytokine / inflammatory marker measurements in plasma samples for the purposes of quantitating one of the secondary outcomes in the CANVAS clinical trial.

REAGENTS:

		Product No
▪ ProcartaPlex Human Basic Kit	Ebioscience	EPX010-10420-901
▪ ProcartaPlex Human CRP Simplex	Ebioscience	EPX010-10288-901
▪ ProcartaPlex Human IFN-gamma Simplex	Ebioscience	EPX010-10228-901
▪ ProcartaPlex Human IL-10 Simplex	Ebioscience	EPX010-10215-901
▪ ProcartaPlex Human IL-17A Simplex	Ebioscience	EPX010-12017-901
▪ ProcartaPlex Human IL-2 Simplex	Ebioscience	EPX010-10221-901
▪ ProcartaPlex Human IL-6 Simplex	Ebioscience	EPX010-10213-901
▪ ProcartaPlex Human TNF-alpha Simplex	Ebioscience	EPX010-10223-901

MATERIALS & METHODS:

- Disposable Gilson Pipette Tips
- Full Set of Calibrated Pipettes
- Microbiological Safety Cabinet
- Distilled water
- Hand-held magnetic plate washer

WORK INSTRUCTION

In addition to this work instruction consult also the Ebioscience protocol for multiplex immunoassays at: http://ebioscience.instant.at/bm_products/MAN/ProcartaPlex%20Human.pdf

1. Prepare the reagents. Dilute the Wash Buffer Concentrate (10x) 1:10 with distilled water.

2. Prepare the antigen standards as per manufacturer's instructions. Centrifuge the antigen standard vial at 2000 x g for 10 sec. Add 250uL of Universal Assay Buffer into the vial. Vortex gently for 30 sec and incubate on ice for 30 minutes.

3. Prepare a 4 fold serial dilution of the antigen standard according to the manufacturer's instructions using the PCR 8-tube strip provided, in order to obtain a 7-point standard curve for the assay panel. Add 200uL of the reconstituted antigen standard mix into the first strip tube and label the tube as Standard 1. Add 150uL Universal Assay Buffer into tubes 2-7 and label accordingly. Transfer 50uL of the reconstituted antigen standards from tube 1 to tube 2. Mix by pipetting up and down for a total of 10 times. After changing the pipette tip, transfer 50uL of the mixed standards from tube 2 to tube 3 and mix as above. Repeat this for the rest of the tubes. Add Universal Assay Buffer to tube 8 which will serve as blank. Keep on ice until ready to use.

4. Prepare 1x detection antibody mixture as per manufacturer's instructions. Each simplex kit contains Detection Antibody Concentrate (50x). Prepare the Detection Antibody Mixture (1x) by combining the Detection Antibody Concentrates (50x) of each simplex kit taking into account the following principles: each well uses 25uL of the Working Detection Antibody Mixture (1x); prepare an appropriate volume of Working Detection Antibody Mixture by combining the concentrated Detection Antibody (50x) of all simplex kits to be plexed with the Detection Antibody Diluent; scale according to the number of assays to be run and include 25% overage; when calculating number of wells, consider the number of wells needed to include the standard curves, blanks and samples.

5. Prepare the 96-well flat bottom plate (included in the basic kit) as per the manufacturer's instructions. Mix the antibody magnetic beads that are to be plexed as follows.

6. Place the 96-well flat bottom plate onto the hand-held magnetic washer and securely lock into place. Vortex each of the antibody magnetic beads for 30 sec and add 5mlof each bead mixture to an appropriate vial. Vortex the antibody magnetic beads for 30 sec. Add the appropriate volume of antibody magnetic beads to each well (300uL to each well if 6 different antibody magnetic beads have been mixed). Wait 2 minutes to allow the Antibody Magnetic Beads to accumulate on the bottom of each well. Remove the liquid in the wells by quickly inverting the Hand-held magnetic plate washer and plate assembly over a sink or waster container. Blot the inverted assembly onto several layers of paper towels to remove any residual solution.

7. Wash antibody magnetic beads using the hand-held magnetic plate washer as per the manufacturer's instructions. Add 150uL of 1x Wash Buffer into each well. Wait 30 sec to allow the antibody magnetic beads to accumulate on the bottom of each well. Remove the wash buffer in the wells by quickly inverting the hand-held magnetic washer and plate assembly over a sink or waster contained. Blot the inverted assembly over several layers of paper towels to remove any residual solution.

8. Prepare the clinical trial samples. Thaw plasma samples required. Use clinical trial samples neat for the plexed assay: IFN-γ, TNF-α, IL-2, IL-6, IL-10, IL-17. Prepare a 1:500 dilution for the CRP assay.

9. Add standards and samples. First, add 25uL of Universal Assay Buffer into each well. Next add 25uL of standards or samples into the appropriate dedicated wells. Add 25uL Universal Assay Buffer to the blank wells.

10. Seal the 96-well plate using the seal provided in the basic kit. Remove the plate from the hand-held magnet and cover the plate with the black microplate lid provided in the basic kit to protect from light. Shake the 96-well plate at 500rpm for 2 hours at room temperature.

11. Insert the plate in the hand-held magnet and wait 2 minutes to allow the antibody magnetic beads to accumulate on the bottom of the well. Carefully remove the plate seal. Remove the solution in the wells as in point 6. Wash twice as in point 7.

12. Add the working detection antibodies mixture. Add 25uL of the mixture into each well. Seal the plate with a new plate seal. Remove the plate from the hand-held magnet and cover with the black microplate lid. Shake at 500 rpm for 30 minutes at room temperature.

13. Wash twice as in point 11.

14. Add streptavidin – PE (SA-PE). Add 50uL of SA-PE solution into each well. Seal the plate with a new plate seal. Remove the plate from the hand-held magnet and cover the plate with the black microplate lid. Shake at 500 rpm for 30 minutes at room temperature.

15. Wash twice as in point 11.

16. Prepare the plate for analysis. Add 120uL of Reading Buffer provided into each well. Seal the plate with a new plate seal. Remove the plate from the hand-held magnet and cover the plate with the black microplate lid. Shake at 500 rpm for 5 minutes at room temperature.

17. Remove the plate seal prior to reading on a Biorad Luminex 200 instrument.

8 Appendix 2:

Luminex Assay Record

Date of Experiment _____

Type of Experiment (i.e. clinical trial sample) _____

Unique Patient ID numbers and time-points _____

_____

_____

_____

_____

Reagents used:

ProcartaPlex Human CRP Simplex	Lot_____	Expiry Date_____
ProcartaPlex Human IFN-gamma Simplex	Lot_____	Expiry Date_____
ProcartaPlex Human IL-10 Simplex	Lot_____	Expiry Date_____
ProcartaPlex Human IL-17A Simplex	Lot_____	Expiry Date_____
ProcartaPlex Human IL-2 Simplex	Lot_____	Expiry Date_____
ProcartaPlex Human IL-6 Simplex	Lot_____	Expiry Date_____
ProcartaPlex Human TNF-alpha Simplex	Lot_____	Expiry Date_____

Pipettes used:

_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____
_____	Calibration due on _____

9 Appendix 3:

COEFFICIENT OF VARIATION (CV) VALUES FOR INTRA AND INTER-ASSAY VARIABILITY PROVIDED BY THE MANUFACTURER (EBIOSCIENCE)

Analyte	Intra-Assay Variability CV (%)	Inter-Assay Variability CV (%)
IFN-γ	3.6	5.5
TNF-α	7.2	6.1
IL-2	6.2	5.1
IL-6	6.2	4.5
IL-10	3.1	2.3
IL-17	6.5	6.0
hsCRP	6.6	9.6

Source: Ebioscience

Appendix 8 Case Report Form

WTCRF 1246

Patient Study ID .....
Patient Initials .....

UNIVERSITY OF BIRMINGHAM

University Hospital Birmingham NHS Foundation Trust

CASE REPORT FORM
CLINICAL TRIAL

CANVAS

CMV Modulation of the Immune System in **AN**CA-Associated **VAS**culitis

Principal Investigator: Professor Lorraine Harper
EudraCT Number: 2012-001970-28
ClinicalTrials.gov Identifier: NCT01633476
CRF Version Number: 1.0

Supported by wellcome trust

Please complete this Case Report Form (CRF) at the end of the patient's involvement in the clinical trial using relevant information from the patient flowsheets.

In addition, please collate to the end of the CRF the forms listed below from the patient flowsheets as they form part of the CRF. Please ensure that the concomitant medications and adverse event log forms have been scanned onto the electronic patient record before collating to the CRF.

1. Cardiovascular Risk Factors Data Capture Form
2. Concomitant Medications Log
3. Adverse Event Log

Patient Study ID .....
Patient Initials .....

Age at study commencement: ……..

Gender: M / F (please circle)

Patient fulfils the study inclusion / exclusion criteria as evidenced by a signed eligibility criteria form in the patient flowsheet

Yes / No (please circle)

Visit 1 – Visit 14

FILL IN THE TABLE OVERLEAF

IF PATIENT WAS RANDOMISED TO CONTROL PLEASE ENSURE FIELDS ARE FILLED WITH *N/A* WHERE APPROPRIATE

WTCRF 1246

Patient Study ID .....
Patient Initials .....

	Date of Visit dd/mm/yyyy	Attendance 0 – Did not attend 1 - Attended	$CD4^{+}CD28^{-}$ %	Randomisation Outcome 0 – Control 1 - Treatment	CMV PCR Result (Blood)	CMV PCR Result (Urine)	Dose of valaciclovir prescribed	Did the patient take at least 80% of the prescribed medication during the previous month? 0 – No 1 – Yes	Comments
Visit 1 (Pre-Baseline)									
Visit 2 (Baseline – Month 0)									
Visit 3 (Month 1)									
Visit 4 (Month 2)									
Visit 5 (Month 3)									
Visit 6 (Month 4)									
Visit 7 (Month 5)									
Visit 8 (Month 6)									
Visit 9 (Month 7)									
Visit 10 (Month 8)									
Visit 11 (Month 9)									
Visit 12 (Month 10)									
Visit 13 (Month 11)									
Visit 14 (Month 12)									

WTCRF 1246

Patient Study ID .....
Patient Initials .....

OFF-STUDY SECTION

Date Off Study: __ __ / __ __ / __ __ __ __ *(DD/MM/YYYY)*
Date Last Study Medication Taken: __ __ / __ __ / __ __ __ __ *(DD/MM/YYYY)*
Reason Off Study (Please mark only the primary reason. Reasons other than Completed Study require explanation next to the response)
☐ Completed study
☐ AE/SAE _____
☐ Lost to follow-up _____
☐ Non-compliant participant_____
☐ Concomitant medication_____
☐ Medical contraindication_____
☐ Withdraw consent _____
☐ Death _____
☐ Other _____

NOW COLLATE TO THE CRF THE FORMS OUTLINED IN PAGE 2

PRINCIPAL INVESTIGATOR'S SIGN OFF

Principal Investigator's Signature Statement:

I have reviewed this CRF and confirm that, to the best of my knowledge, it accurately reflects the study information obtained for this participant. All entries were made by a person under my supervision who has signed the delegation and signature log.

Principal Investigator's Name: _____

Principal Investigator's Signature:_____

Date: __ __ / __ __ / __ __ __ __ (dd/ mm/yyyy)

ONCE SIGNED, NO FURTHER CHANGES CAN BE MADE TO THIS CRF WITHOUT A SIGNED DATA QUERY FORM.

APPENDIX

1. Cardiovascular Risk Factors Data Capture Form
2. Concomitant Medications Log
3. Adverse Event Log

Appendix 9 Development Safety Update Report

CMV Modulation of the Immune System in ANCA Associated Vasculitis (CANVAS)

Development Safety Update Report (DSUR)

Report Number:	3	This is the third DSUR for the use of valaciclovir in the CANVAS trial.

IMP(s):	Valaciclovir Hydrochloride
Reporting Period:	20th November 2014 – 20th November 2015
Report Date:	25th January 2016
Sponsor:	University of Birmingham Edgbaston, Birmingham, West Midlands, B14 2TT
Sponsor Ref Number:	RG_12-142
EudraCT Number:	2012-001970-28
REC Reference Number:	12/YH/0377

Name: Dr Dimitrios Chanouzas

Trial Role

Clinical Coordinator / Research Fellow

Signature:

Date: 25 / Jan / 2016

This report was prepared by Dr Dimitrios Chanouzas on behalf of the Sponsor and contains confidential information.

Executive Summary

IMP(s): Valaciclovir	Valaciclovir Hydrochloride 500mg Film Coated Tablets Valaciclovir, an antiviral, is the L-valine ester of acyclovir. Acyclovir is a purine (guanine) nucleoside analogue.
Treatment Arms:	Open Label Trial Treatment Arm: Valaciclovir 2g QDS (dose adjusted depending on renal function) for a total duration of 6 months
Indication and Population:	To determine whether valaciclovir has the potential to prevent CMV-mediated adverse modulation of the immune system in patients with ANCA-Associated Vasculitis
Estimated Cumulative Exposure:	N/A
Marketing Approval	Valaciclovir has a Marketing Authorisation however the University of Birmingham are not the holders of the MA and therefore the CANVAS trial team are not aware of the worldwide approval statues

Summary and Conclusions:

At the time of writing of this report 4 patients recruited to the study and randomised to the treatment arm have experienced a total of 6 serious adverse events. There have not been any SUSARs to report.

However none of those SAEs experienced during this reporting period were related to the study drug (valaciclovir), therefore there are no SAR to report.

The risks and side effect profile of valaciclovir therefore remain consistent with the information contained in the CTA application for this study.

Table of contents

1. Introduction 5
2. Worldwide Marketing Approval Status 5
3. Actions Taken in the Reporting Period for Safety Reasons 5
4. Changes to Reference Safety Information 6
5. Inventory of Clinical Trials Ongoing and Completed during the Reporting Period 6
6. Estimated Cumulative Exposure 7
6.1 Cumulative Subject Exposure in the Development Programme 7
6.2 Patient Exposure from Marketing Experience 7
7. Data in Line Listings and Summary Tabulations 7
7.1 Reference Information 7
7.2 Line Listings of Serious Adverse Reactions during the Reporting Period 7
7.3 Cumulative Summary Tabulations of Serious Adverse Events 7
8. Significant Findings from Clinical Trials during the Reporting Period 8
8.1 Completed Clinical Trials 8
8.2 Ongoing Clinical Trials 8
8.3 Long-term Follow-up 8
8.4 Other Therapeutic Use of Investigational Drug 8
8.5 New Safety Data Related to Combination Therapies 8
9. Safety Findings from Non-interventional Studies 8
10. Other Clinical Trial/Study Safety Information 8
11. Safety Findings from Marketing Experience 8
12. Non-clinical Data 8
13. Literature 8
14. Other DSURs 8
15. Lack of Efficacy 9
16. Region-Specific Information 9
Cumulative summary tabulation of Serious Adverse Reactions 9
List of subjects who died during the reporting period 9
17. Late-Breaking Information 9
18. Overall Safety Assessment 9
18.1. Evaluation of the Risks 9
18.2 Benefit-risk Considerations 9
19. Summary of Important Risks 9
20. Conclusions 9

Appendices to the DSUR

- ☐ Appendix 1: Current and any revisions to the Reference Safety Information (RSI)
- ☐ Appendix 2: Cumulative Table of Important Regulatory Advice (refer to Section 3)
- ☐ Appendix 3: Status of Ongoing and Completed Clinical Trials (refer to Section 5)

- ☐ Appendix 4: Cumulative Summary Tabulations (refer to Section 6)
- ☐ Appendix 5: A line listing of all Serious Adverse Reactions
- ☐ Appendix 6: A cumulative tabulation of all Serious Adverse Events
- ☐ Appendix 7: Scientific Abstracts (if relevant)
- ☐ Appendix 8: A cumulative tabulation of Serious Adverse Reactions

The above Appendices have been deleted from this report where there are no data available or no SAR / SUSAR reported during this reporting period.

1. Introduction

Report Number:	3	This is the third DSUR for the use of valaciclovir in the CANVAS trial.

Reporting Period:	20th November 2014 – 20th November 2015
Report Date:	25th January 2016
CTA Approval Date:	20th November 2012
IMP(s):	Valaciclovir Hydrochloride 500mg Film Coated Tablets Valaciclovir, an antiviral, is the L-valine ester of acyclovir. Acyclovir is a purine (guanine) nucleoside analogue.
Treatment Arms:	Open Label Trial Treatment Arm: Valaciclovir 2g QDS (dose adjusted depending on renal function) for a total duration of 6 months
Indication and Population:	To determine whether valaciclovir has the potential to prevent CMV-mediated adverse modulation of the immune system in patients with ANCA-Associated Vasculitis
Scope:	This DSUR covers the CANVAS trial

The University of Birmingham does not hold the Manufacturing or Marketing Authorisations for the IMP(s) listed in this DSUR and therefore the CANVAS team does not have access to some of the requested data. Where information is not available the response 'Data not available' or 'Not applicable' is provided.

2. Worldwide Marketing Approval Status

Valaciclovir has a Marketing Authorisation however the University of Birmingham are not the holders of the MA and therefore the CANVAS trial team are not aware of the worldwide approval statues

3. Actions Taken in the Reporting Period for Safety Reasons

There have not been any actions taken in the reporting period for safety reasons.

During Reporting Period

Action	Reason	Update
No Action Taken	*N/A*	*N/A*

4. Changes to Reference Safety Information (RSI)

No Changes

RSI for Reporting Period:

Summary of Product Characteristics (SmPC) as supplied in the CTA Application

Changes to the RSI during the reporting period:

No changes to the RSI were made during the reporting period

5. Inventory of Clinical Trials Ongoing and Completed during the Reporting Period

EudraCT No.	2012-001970-28
Phase	Phase II
Status	Ongoing
Country	UK
Trial title	CMV Modulation of the Immune System in ANCA-Associated Vasculitis (CANVAS)
Trial design	Randomised Open Label
Regimen and dose	Valaciclovir 2g QDS (dose adjusted according to renal function) for 6 months
Trial population	Patients with stable ANCA-Associated Vasculitis > 18 years old
Date of clinical start (First Visit First Patient)	01.08.2013
Planned enrolment	50 patients
Subject exposure (No. recruited patients)	19 patients randomised to treatment arm (40 patients recruited in total)

6. Estimated Cumulative Exposure

6.1 Cumulative Subject Exposure in the Development Programme

Cumulative exposure for the CANVAS trial is summarised below:

Treatment	19
Valaciclovir	19
Total	40 patients in total recruited to trial

6.2 Patient Exposure from Marketing Experience

Data not available.

7. Data in Line Listings and Summary Tabulations

7.1 Reference Information

Coding document used to record Adverse Events	National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0

RSI used to assess expectedness

	Effective from:	**Effective to:**
SmPC as submitted in CTA Application	20.11.2012	End of study

7.2 Line Listings of Serious Adverse Reactions during the Reporting Period

There have been no Serious Adverse Reactions during the reporting period

7.3 Cumulative Summary Tabulations of Serious Adverse Events

See Appendix 6

8. Significant Findings from Clinical Trials during the Reporting Period

8.1 Completed Clinical Trials

Not applicable. No relevant completed trials included within this DSUR.

8.2 Ongoing Clinical Trials

Not applicable.

8.3 Long-term Follow-up

Not applicable.

8.4 Other Therapeutic Use of Investigational Drug

Not applicable; this DSUR has been prepared for the trial described in the scope (section 1).

8.5 New Safety Data Related to Combination Therapies

Not applicable; this DSUR has been prepared for the trial described in the scope (section 1).

9. Safety Findings from Non-interventional Studies

No information from non-interventional studies has become available to the CANVAS Trial Team during this reporting period.

10. Other Clinical Trial/Study Safety Information

No information from clinical trials/studies has become available to the CANVAS Trial Team during this reporting period.

11. Safety Findings from Marketing Experience

No information regarding safety findings from marketing experience has been brought to the attention of trial team during this reporting period.

12. Non-clinical Data

No information from non-clinical studies has become available to the trial team during this reporting period.

13. Literature

No new literature affecting the risk and side effect profile of valaciclovir has become available to the trial team during this reporting period.

14. Other DSURs

Valaciclovir is manufactured/ marketed by Ranbaxy UK Limited and the CANVAS Trial Team do not have access to the any other DSUR.

15. Lack of Efficacy

Not applicable.

16. Region-Specific Information

Not applicable.

17. Late-Breaking Information

Not applicable.

18. Overall Safety Assessment

The risks and side effect profile of valaciclovir remain consistent with the information contained in the CTA application for this study.

18.1. Evaluation of the Risks

There have been no newly identified safety issues during this reporting period.

18.2 Benefit-risk Considerations

The risk benefit considerations of using valaciclovir in the context of the CANVAS clinical trial have not changed from those detailed in the CTA Application.

19. Summary of Important Risks

A risk of acute kidney injury (reduction in renal function) was identified during the second reporting period (20/11/2013 to 20/11/2014; see second DSUR). However this is a well-known risk / side effect of valaciclovir and does not constitute new information. There have been no further such events in this current reporting period.

Importantly there have been no risks identified from the reported SAEs during this reporting period as no SAEs were related to the study drug valaciclovir.

20. Conclusions

The risks remain fairly consistent with the experience described in the CTA Application, and we conclude that the information obtained in this reporting period justifies continuation of the trial.

Appendix 6: A cumulative tabulation of all Serious Adverse Events

CTCAE Category	Adverse Event Term	Number of Adverse Events	
		Treatment Arm	Control Arm
Infections and Infestations	Lung Infection	5 (1246SAE001, 1246SAE005, 1246SAE007, 1246SAE011, 1246SAE012)	2 (1246SAE002, 1246SAE006)
	Abdominal infection		1 (1246SAE003)
	Sub-total 8		
Musculoskeletal and connective tissue disorders	Arthritis	1 (1246SAE004)	
	Sub-total 1		
Cardiac disorders	Palpitations		1 (1246SAE008)
	Sub-total 1		
Respiratory, thoracic and mediastinal disorders	Epistaxis		1 (1246SAE009)
	Thromboembolic event		1 (1246SAE010)
	Sub-total 2		

Appendix 10 REC and MHRA approvals