# Vaccination against Hepatitis C - Characterising the host immune response and its implications for a successful therapeutic vaccine

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# ABSTRACT

## VACCINATION AGAINST HEPATITIS C -CHARACTERISING THE HOST IMMUNE RESPONSE AND ITS IMPLICATIONS FOR A SUCCESSFUL THERAPEUTIC VACCINE

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This thesis explores the first-ever administration of a prime/boost combination of adenovirus and MVA vector based vaccines that target hepatitis C virus (HCV) infection to humans. Therapeutic application of these vaccines in patients with chronic HCV infection is first detailed, together with an interrogation of the immune responses generated. Vaccination safely induces novel T cells that target HCV immunogen in 5/11 vaccinated patients. However, assessment of circulating virus reveals significant sequence differences between autologous virus and the corresponding peptide in the vaccine immunogen. Further analysis shows that T cells induced by vaccination are poorly cross-reactive against autologous virus epitopes. Compared with vaccination in healthy individuals, T cell responses are reduced in their frequency, magnitude and breadth in HCV infected patients who receive vaccination, either alone or in combination with standard HCV treatment (pegylated interferon-α (PEG-IFN)/ribavirin).

To help explain the reason for attenuated vaccine responses in HCV infected patients, I next explored baseline and PEG-IFN/ribavirin induced differences in circulating lymphocyte subpopulations. At baseline, high proportions of regulatory T cells and $NK^{bright}$ cells suggest both adaptive and innate immunity is altered by chronic HCV infection. During the first 28 days of therapy, complex changes in the activation, differentiation status and cytokine profiles of circulating lymphocytes are observed over time. Intriguingly, PEG-IFN causes a transient but significant decrease in circulating lymphocytes that express CXCR3, a liver homing chemokine receptor.

Finally, the hypothesis that HCV therapy induces CXCR3 mediated hepatic sequestration of lymphocytes was explored. In-vivo, PEG-IFN caused a transient but profound elevation of plasma CXCL10 (a CXCR3-binding chemokine) and this correlated with declines in circulating $CXCR3^+$ $CD8^+$ T cells in HCV infected patients. Although in-vitro experiments show IFN-α drives hepatocytes to produce CXCL10, it also causes lymphocytes to down-regulate CXCR3. Unfortunately, exogenous IFN-α does not objectively demonstrate hepatic enrichment of $CXCR3^+$ lymphocytes in a murine model.

## PUBLICATIONS RELATED TO THIS WORK

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## PRESENTATIONS & PUBLIC APPEARANCES

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  Poster: J.S. Halliday, N. Ramamurthy, C. Willberg, J. Ussher, J. Phillips, L. Swadling, C. Cosgrove, P. Klenerman, E. Barnes *'CXCL10 release driven by interferon-α suggests a role for lymphocyte recruitment in HCV clearance'*
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  II. *'Viral Vaccines'*
     Masters of Immunology course, Oxford University
  III. '*Vaccines against viral infection'*
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  IV. 'Vaccination against hepatitis C
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2011

- EASL (European Association for the study of liver disease), Berlin
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- Lectures/Presentations given
  I. *'Vaccination for hepatitis C'*
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  II. Oxford/Cambridge GUT club
  III. Oxford/Wessex GUT club - awarded best speaker
  IV. Hepatitis C public lecture, Oxford

# TABLE OF CONTENTS


**Chapter 1 Introduction ..... 16**

1.1 HEPATITIS C INFECTION ..... 16
1.1.1 AETIOLOGY ..... 17
1.1.2 CLINICAL FEATURES AND DIAGNOSIS OF HCV INFECTION ..... 20
1.1.3 CURRENT MEDICAL THERAPY FOR HCV (INCLUDING MECHANISMS OF ACTION) ..... 22
1.2 HOST IMMUNE RESPONSES TO HEPATITIS C INFECTION ..... 33
1.2.1 RELEVANCE TO VACCINE DEVELOPMENT ..... 33
1.2.2 ANTI-HCV SPECIFIC T CELL RESPONSES ..... 35
1.2.3 HUMORAL IMMUNITY TO HCV ..... 38
1.2.4 INNATE IMMUNITY ..... 39
1.3 VACCINATION AGAINST HEPATITIS C – BACKGROUND ..... 41
1.3.1 THE IDEAL VACCINE – REQUIREMENTS AND CHALLENGES ..... 41
1.3.2 VACCINE APPROACHES: CURRENT STATUS ..... 43
1.4 SUMMARY OF VACCINE APPROACHES AND OXFORD HCV VACCINE PROGRAM ..... 57
1.5 THERAPEUTIC HCV VACCINE TRIAL (HCV003) ..... 58
1.5.1 WHAT VACINE IMMUNOGEN SHOULD BE USED? ..... 58
1.5.2 WHICH VIRAL VECTOR SHOULD BE USED TO DELIVER VACCINE IMMUNOGEN? ..... 60
1.5.3 TO WHOM SHOULD THE VACCINE BE ADMINISTERED AND ACCORDING TO WHAT SCHEDULE? ..... 64
1.5.4 WHAT ARE THE POTENTIAL RISKS OF VACCINATION/TRIAL PARTICIPATION? ..... 65
1.5.5 WHAT SHOULD THE PRIMARY OBJECTIVES OF THE THERAPEUTIC ARMS OF THE HCV003 TRIAL BE? ..... 66
1.5.6 ETHICAL APPROVAL ..... 66
1.6 THE HOST IMMUNE RESPONSE TO STANDARD THERAPY TRIAL (THIRST TRIAL) ..... 67
1.6.1 RATIONALE FOR STUDY ..... 67
1.6.2 ETHICAL APPROVAL FOR THE THIRST STUDY ..... 69
1.7 SUMMARY OF INTRODUCTION ..... 69

**Chapter 2 Materials and Methods ..... 70**

2.1 ESTABLISHING COHORTS OF PATIENTS AND CONTROLS ..... 70
2.1.1 HCV003 TRIAL ..... 70
2.1.2 THE HOST IMMUNE RESPONSE TO STANDARD THERAPY TRIAL (THIRST TRIAL) ..... 73
2.2 VIRAL LOAD AND FULL BLOOD COUNT DETERMINATION ..... 73
2.3 PLASMA PREPARATION ..... 73
2.4 LYMPHOCYTE COLLECTION/FREEZING: METHOD USED FOR THIRST AND HCV003 TRIALS ..... 74
2.4.1 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) PREPARATION ..... 74
2.4.2 FREEZING/THAWING PBMCS ..... 74
2.5 HCV SPECIFIC PEPTIDE POOLS ..... 75
2.6 ELISPOT DETERMINATION OF IFN-γ PRODUCTION FOLLOWING HCV PEPTIDE STIMULATION ..... 75
2.6.1 IFN-γ ELISPOT PROTOCOL ..... 75
2.6.2 DEFINITION OF POSITIVE IFN-γ ELISPOT RESPONSE ..... 76
2.6.3 FINE MAPPING OF POSITIVE IFN-γ ELISPOT RESPONSES ..... 77
2.6.4 PROLIFERATION ASSAYS ..... 77
2.6.5 SUMMARY OF T CELL ASSAYS PERFORMED IN THE HCV003 TRIAL ..... 78
2.7 SEQUENCING OF CIRCULATING VIRUS IN PATIENTS WITH CHRONIC HCV INFECTION ..... 79
2.7.1 RNA EXTRACTION ..... 79
2.7.2 POLYMERASE CHAIN REACTION (PCR) ..... 79
2.7.3 PCR PRODUCT EXTRACTION ..... 80
2.7.4 DIRECT SEQUENCING ..... 80
2.8 ANTIBODY STAINING FOR FLOW CYTOMETRY ASSESSMENT OF CELL PHENOTYPE ..... 82

2.8.1 DETERMINING APPROPRIATE ANTIBODY CONCENTRATION ..... 82
2.8.2 CELL PREPARATION ..... 82
2.8.3 STIMULATION OF CELLS FOR FUNCTIONAL ASSESSMENT OF CYTOKINE PRODUCTION ..... 82
2.8.4 SURFACE STAINING OF PBMCS FOR FLOW CYTOMETRY ..... 83
2.8.5 INTRACELLULAR STAINING FOR FUNCTIONAL AND ICS FLOW CYTOMETRY PANELS ..... 83
2.8.6 FACS ANALYSIS ..... 84
2.9 IL-28 GENOTYPING WITH REAL-TIME qPCR ..... 87
2.9.1 DNA EXTRACTION ..... 87
2.9.2 IL-28 GENOTYPING ..... 89
2.10 ASSESSMENT OF CXCL10 PRODUCTION BY HEPATOCYTES IN-VITRO FOLLOWING IFN-α STIMULATION ..... 91
2.10.1 CELL STIMULATION AND RNA EXTRACTION PROTOCOL ..... 91
2.11 ASSESSMENT OF CXCL10 PRODUCTION BY qPCR ..... 92
2.11.1 REVERSE TRANSCRIPTION TO cDNA ..... 92
2.11.2 QUANTITATIVE PCR USING ROCHE LIGHT CYCLER 480 ..... 93
2.12 ELISA ASSAY FOR CXCL10 ..... 94
2.12.1 REAGENT PREPARATION ..... 94
2.12.2 ELISA ASSAY PROCEDURE ..... 95
2.13 MURINE EXPERIMENTS ..... 96
2.13.1 LYMPHOCYTE EXTRACTION FOR MOUSE EXPERIMENT ..... 97
2.13.2 LYMPHOCYTE QUANTIFICATION ..... 98
2.14 STATISTICAL METHODS ..... 99

**Chapter 3 Therapeutic vaccination for hepatitis C virus infection ..... 100**

3.1 PRIMARY HYPOTHESIS ..... 100
3.2 PRIMARY OBJECTIVES ..... 100
3.3 KEY QUESTIONS ARISING FROM PRIMARY OBJECTIVES ..... 100
3.4 CLINICAL PARAMETERS ..... 101
3.4.1 BASELINE DEMOGRAPHICS ..... 101
3.4.2 CLINICAL PROGRESS DURING PEG-IFN/RIBAVIRIN THERAPY ..... 103
3.5 KEY QUESTION 1 - Is it safe to vaccinate patients who have chronic HCV infection with ChAd3-NSmut and MVA-NSmut? ..... 104
3.5.1 ChAd3-NSmut PRIME VACCINATION ..... 104
3.5.2 MVA-NSmut BOOST VACCINATION ..... 104
3.6 KEY QUESTION 2 - Prior to vaccination, can T cells that recognise HCV non-structural peptides be detected in HCV infected trial participants, and if so, what do they target? ..... 106
3.6.1 BASELINE IFN-γ ELISPOT RESPONSES TO HCV NS PEPTIDES INCLUDED IN VACCINE IMMUNOGEN ..... 106
3.6.2 WHAT IS THE AUTOLOGOUS VIRAL SEQUENCE IN REGIONS CORRESPONDING TO T CELL EPITOPES DETECTED BY IFN-γ ELISpot AT BASELINE? ..... 108
3.7 KEY QUESTION 3 - Does ChAd3-NSmut and MVA-NSmut vaccination successfully induce T cells in HCV infected patients? ..... 110
3.7.1 CAN T CELLS BE INDUCED BY VACCINATION IN HEALTHY VOLUNTEERS? ..... 110
3.7.2 CAN T CELLS BE INDUCED BY VACCINATION IN HCV INFECTED PATIENTS? ..... 112
3.7.3 VACCINE RESPONDERS – IS T CELL INDUCTION LIMITED TO PEPTIDES WITHIN THE VACCINE? ..... 117
3.7.4 MAPPING OF T CELL RESPONSES IN HCV INFECTED PATIENTS ..... 119
3.8 KEY QUESTION 4 – Do vaccine induced T cells effectively target circulating virus? ..... 121
3.8.1 IS THERE A RELATIONSHIP BETWEEN VACCINE INDUCED IFN-γ ELISPOT RESPONSES AND HCV VIRAL LOAD? ..... 122
3.8.2 WHAT IS THE SEQUENCE OF CIRCULATING VIRUS AT SITES THAT CORRESPOND TO VACCINE INDUCED T CELL EPITOPES? ..... 124
3.8.3 HOW VARIABLE ARE HCV SEQUENCES AT IMMUNOGENIC EPITOPES TARGETED BY VACCINE INDUCED T CELLS? ..... 126

3.8.4 DO VACCINE INDUCED T CELLS TARGET EPITOPE VARIANTS THAT CORRESPOND TO CIRCULATING VIRUS? 128
3.8.5 WHAT IS THE PHENOTYPE OF VACCINE INDUCED T CELLS? 130
3.8.6 WHAT IS THE PROLIFERATIVE CAPACITY OF VACCINE INDUCED T CELLS IN HCV INFECTED PATIENTS? 135
3.8.7 WHAT IS THE CYTOKINE PROFILE OF VACCINE INDUCED T CELLS? 136
3.9 SUMMARY DISCUSSION 138
3.10 CONCLUSION 145

**Chapter 4 The Host Immune Response to Standard Therapy during chronic Hepatitis C virus infection (THIRST trial) 147**

4.1 INTRODUCTION 147
4.2 PRIMARY HYPOTHESES 147
4.3 PRIMARY OBJECTIVES 148
4.4 KEY QUESTIONS 148
4.5 DEMOGRAPHICS AND CLINICAL DATA 149
4.5.1 HCV INFECTED PATIENTS ENROLLED IN THIRST TRIAL 149
4.5.2 HEALTHY VOLUNTEERS 154
4.6 KEY QUESTION 1 - Does HCV infection or PEG-IFN/ribavirin therapy change lymphocyte subsets in circulation? 155
4.6.1 $CD3^+$, $CD4^+$ AND $CD8^+$ T CELLS 155
4.6.2 REGULATORY T CELLS 158
4.6.3 B CELLS 160
4.6.4 $CD161^+$ T CELLS 161
4.6.5 NK CELLS 163
4.6.6 DISCUSSION RELATING TO CHANGES IN CIRCULATING LYMPHOCYTE SUBSETS DURING THERAPY 166
4.7 KEY QUESTION 2 – Does chronic HCV infection or PEG-IFN/ribavirin therapy change the cytotoxicity of circulating lymphocytes? 173
4.7.1 PERFORIN AND GRANZYME B EXPRESSION 173
4.7.2 DISCUSSION RELATING TO CYTOTOXIC CAPACITY 175
4.8 KEY QUESTION 3 – Does HCV infection or PEG-IFN/ribavirin therapy change the homing phenotype of PBMCs? 177
4.8.1 CCR6 EXPRESSION 178
4.8.2 CXCR3 EXPRESSION 180
4.8.3 DIFFERENTIAL EXPRESSION OF CCR6 AND CXCR3 BY T CELLS 183
4.8.4 DISCUSSION RELATING TO LYMPHOCYTE HOMING 186
4.9 KEY QUESTION 4 - Does HCV infection or PEG-IFN/ribavirin therapy change cytokine production by PBMCs? 189
4.9.1 TNF-α 189
4.9.2 IL-17 191
4.9.3 IFN-γ 193
4.9.4 IL-2 194
4.9.5 DISCUSSION RELATING TO CHANGES IN LYMPHOCYTE CYTOKINE PROFILE 195
4.10 KEY QUESTION 5 – Does HCV infection or PEG-IFN/ribavirin therapy cause lymphocyte activation? 200
4.10.1 HLA-DR EXPRESSION 200
4.10.2 CD69 EXPRESSION 202
4.10.3 CD38 EXPRESSION 203
4.10.4 DISCUSSION RELATING TO LYMPHOCYTE ACTIVATION 204
4.11 KEY QUESTION 6 – Does HCV infection or PEG-IFN/ribavirin therapy alter the differentiation state of circulating lymphocytes? 208
4.11.1 MEMORY PHENOTYPES: CCR7/45RA 208
4.11.2 CD57 EXPRESSION 213
4.11.3 CD62L EXPRESSION 214

4.11.4 DISCUSSION RELATING TO LYMPHOCYTE DIFFERENTIATION DURING HCV THERAPY 215
4.12 SUMMARY DISCUSSION OF THIRST STUDY ..... 218
4.13 CONCLUSION ..... 221

**Chapter 5 The role of CXCR3 in lymphocyte recruitment during HCV therapy ..... 223**

5.1 INTRODUCTION ..... 223
5.2 PRIMARY HYPOTHESIS ..... 225
5.3 PRIMARY OBJECTIVE ..... 225
5.4 KEY QUESTIONS ..... 225
5.5 KEY QUESTION 1 - Does IFN-α induce CXCR3-binding chemokines in vivo, and are these linked to changes in CXCR3 expression? ..... 226
5.5.1 EVALUATION OF CXCL10 DURING PEG-IFN/RIBAVIRIN THERAPY ..... 226
5.5.2 CHANGES IN PLASMA CXCL11 DURING PEG-IFN/RIBAVIRIN THERAPY ..... 231
5.5.3 IS THERE A RELATIONSHIP BETWEEN CXCL11 AND CXCL10 INDUCTION? ..... 233
5.5.4 IS THERE A RELATIONSHIP BETWEEN CXCR3-BINDING CHEMOKINES AND HOST IL-28 STATUS? ..... 234
5.6 KEY QUESTION 2: Is the liver a source of CXCR3-binding chemokines induced by IFN-α? ..... 235
5.6.1 Huh7.5 mRNA EXPRESSION FOLLOWING IFN-α STIMULATION ..... 235
5.6.2 CXCL10 PRODUCTION BY Huh7.5 CELLS FOLLOWING IFN-α STIMULATION ..... 235
5.7 KEY QUESTION 3: Does IFN-α cause down-regulation of CXCR3 expression or induce death of CXCR3$^+$ lymphocytes? ..... 236
5.8 KEY QUESTION 4: Does exogenous IFN-α drive enrichment of hepatic CXCR3$^+$ lymphocytes in a murine model? ..... 241
5.8.1 DO CIRCULATING OR ORGAN SEQUESTERED LYMPHOCYTE NUMBERS CHANGE FOLLOWING ADMINISTRATION OF IFN-α IN MICE? ..... 242
5.8.2 DOES CXCR3 EXPRESSION ON MURINE LYMPHOCYTES CHANGE FOLLOWING ADMINISTRATION OF IFN-α? ..... 245
5.8.3 SUMMARY OF MURINE EXPERIMENTAL RESULTS ..... 261
5.9 SUMMARY DISCUSSION ..... 262
5.10 CONCLUSION ..... 268

**Chapter 6 Concluding remarks ..... 269**
**Chapter 7 Appendices ..... 279**
**Chapter 8 References ..... 294**

# LIST OF FIGURES

Figure 1-1; The HCV genome and expressed polyprotein ..... 17
Figure 1-2; Comparison of the phylogenetic trees of HCV, HIV and Hepatitis B virus ..... 19
Figure 1-3 HCV mediated stimulation of endogenous IFN-α production ..... 26
Figure 1-4 The downstream effects of IFN-α, together with mechanisms through which HCV subverts these responses ..... 27
Figure 1-5; Schematic representation of viral/ host immune interactions during acute and chronic HCV infection ..... 35
Figure 1-6; The non-structural (NS) regions of HCV encoded by the vaccine. ..... 59
Figure 1-7; Magnitude and kinetics of T cell responses after vaccination with prime/ boost vaccination in primates (left graph) and healthy volunteers (right graph) ..... 62
Figure 2-1 Summary of T cell assays performed in HCV003 trial ..... 78
Figure 2-2 Fluorescence Scatter Plot for IL-28 genotyping generated by Light Cycler real-time PCR ..... 90
Figure 2-3; Murine experiment protocol summary ..... 96
Figure 3-1; Recruitment and clinical progress of patients enrolled in HCV003 study ..... 103
Figure 3-2; Adverse events following vaccination (with definite, probable or possible causal relationships) ..... 105
Figure 3-3; Summed IFN-γ ELISpot responses to the NS region from ..... 106
Figure 3-4; Magnitude and kinetics of T cell responses after vaccination with ChAd3-NSmut (prime) and MVA-NSmut (boost) in healthy volunteers ..... 111
Figure 3-5; Magnitude of IFN-γ ELISpot responses to NS HCV peptide pools in all patients (Arms B &C) vaccinated with ChAd3-NSmut and MVA-NSmut ..... 112
Figure 3-6; The magnitude and kinetics of T cell responses after vaccination with ChAd3-NSmut (prime) and MVA-NSmut (boost) ..... 113
Figure 3-7; IFN-γ ELISspot responses to NS HCV peptide pools over time in individual patients during PEG-IFN/ribavirin therapy ..... 114
Figure 3-8; Magnitude and kinetics of T cell responses after prime/boost vaccination for patients enrolled in Arm C ..... 115
Figure 3-9; IFN-γ ELISspot responses to NS HCV peptide pools over time in individual patients in Arm C ..... 116
Figure 3-10; Peak change in IFN-γ ELISpot from baseline following boost vaccination in vaccine responders: vaccine peptides vs. non-vaccine peptides ..... 117
Figure 3-11; Longitudinal change in viral load relative to IFN-γ ELISpot response in individual patients in Arm C ..... 122
Figure 3-12; Longitudinal change in viral load relative to IFN-γ ELISpot response in individual patients in Arm B2 ..... 123
Figure 3-13; Sequence variability at vaccine induced T cell epitopes at a population level in Genotype 1 HCV infection defined using Los Alamos database ..... 127
Figure 3-14; T cell responses to peptides corresponding to the vaccine sequence compared with host virus sequence at immunogenic epitopes ..... 128
Figure 3-15; IFN-γ ELISpot responses to vaccine immunogen compared with corresponding autologous viral peptide ..... 129
Figure 3-16; Granzyme A, Granzyme B and perforin expression on pentamer ($NS3_{1406}$) positive T cells after boost vaccination ..... 131
Figure 3-17; CD38, CD127, HLA-DR and PD-1 expression on pentamer ($NS3_{1406}$) positive T cells after boost vaccination ..... 132
Figure 3-18; Method for defining memory phenotypes; CCR7 and CD45RA expression used in combination as illustrated ..... 133
Figure 3-19; Memory phenotype of vaccine induced T cells 1 week after boost vaccination in patients 355 and 360 compared with total CD8+ T cell population ..... 134
Figure 3-20; Memory phenotype of vaccine induced T cells 1 week after boost vaccination in patient 355. CCR7/CD45RA co-expression is demonstrated on this raw FACS plot. ..... 134
Figure 3-21; Proliferative capacity of vaccine induced T cells: 3 healthy volunteers compared with 3 HCV infected patients ..... 135
Figure 3-22; IFN-γ production following peptide pool stimulation of PBMCs collected 1 week after boost vaccination ..... 136

Figure 3-23; Cytokine production by $CD4^+$ and $CD8^+$ T cells isolated from patient 355 before and after boost vaccination following peptide stimulation in-vitro ..... 137
Figure 3-24 Why do vaccine immunogen and viral sequence always differ at immunogenic epitopes in HCV infected vaccine responders? ..... 142
Figure 4-1; THIRST trial recruitment and clinical progress during therapy ..... 151
Figure 4-2; Longitudinal circulating lymphocyte counts during PEG-IFN/ribavirin therapy in HCV infected patients ..... 152
Figure 4-3; HCV viral kinetics during PEG-IFN/ribavirin therapy ..... 153
Figure 4-4; FACS gating strategy used to define $CD3^+$, $CD4^+$ and $CD8^+$ T cells ..... 155
Figure 4-5; $CD3^+$, $CD4^+$ and $CD8^+$ T cell populations in patients with CHC compared with healthy controls ..... 156
Figure 4-6; Longitudinal changes in circulating lymphocyte subpopulations during PEG-IFN/ribavirin therapy ..... 157
Figure 4-7; FACS plots demonstrating gating strategy used to define regulatory T cells ..... 158
Figure 4-8; Baseline differences and longitudinal changes in Tregs during HCV therapy ..... 159
Figure 4-9; Example FACS plot for gating strategy used to define B cells ..... 160
Figure 4-10; Baseline differences and longitudinal changes in B cells during HCV therapy ..... 160
Figure 4-11; FACS plot demonstrating gating strategy used to define $CD161^+$ cells ..... 161
Figure 4-12; Baseline differences and longitudinal changes during HCV therapy in $CD161^+$ $CD4^+$ and $CD8^+$ T cells ..... 162
Figure 4-13; FACS plots demonstrating gating strategies used to define NK cell subsets ..... 163
Figure 4-14; Baseline comparison of total NK cell populations between patients and controls ..... 164
Figure 4-15; $CD56^{bright}$ NK cells as proportions of the NK cell population ..... 164
Figure 4-16; NK cell subpopulations as proportions of total PBMCs - longitudinal changes during HCV therapy in patients compared with controls ..... 165
Figure 4-17; Granzyme B expression by T cells – longitudinal changes during HCV therapy in patients compared with controls ..... 174
Figure 4-18; Perforin expression by T cells – longitudinal changes during HCV therapy in patients compared with controls ..... 174
Figure 4-19; FACs plot demonstrating gating strategy used to define $CXCR3^+$ cells ..... 177
Figure 4-20; FACS plot demonstrating CCR6 gating strategy together with longitudinal change in its expression during therapy ..... 178
Figure 4-21; CCR6 expression by T cells - longitudinal changes during HCV therapy in patients compared with controls ..... 179
Figure 4-22; CCR6 expression by B cells and NK cells; longitudinal changes during HCV therapy in patients compared with controls ..... 180
Figure 4-23; CXCR3 expression by T cells - longitudinal changes during HCV therapy in patients compared with controls ..... 181
Figure 4-24; CXCR3 expression by B cells and NK cells - longitudinal changes during HCV therapy in patients compared with controls ..... 182
Figure 4-25; Differential co-expression of CCR6 and CXCR3 by $CD4^+$ and $CD8^+$ T cells during HCV therapy ..... 183
Figure 4-26; FACS plots demonstrating CXCR3 and CCR6 expression by NK cell subpopulations ..... 185
Figure 4-27; TNF-α production – baseline and longitudinal changes during HCV therapy in HCV infected patients compared with controls ..... 190
Figure 4-28; IL-17 production - baseline and longitudinal changes in during HCV therapy in patients compared with healthy controls ..... 191
Figure 4-29; ΔIL-17 production by $CD4^+$ T cells after 4 weeks therapy (day 28 – day 0) ..... 192
Figure 4-30; Longitudinal changes in IFN-γ production by lymphocytes during therapy in HCV infected patients compared with healthy controls ..... 193
Figure 4-31; Longitudinal changes in IL-2 production by lymphocytes during therapy in HCV infected patients compared with healthy controls ..... 194
Figure 4-32; HLA-DR expression – baseline and longitudinal changes during therapy in HCV infected patients compared with healthy controls ..... 201
Figure 4-33; Longitudinal changes in CD69 expression during therapy in HCV ..... 202
Figure 4-34; Longitudinal changes in CD38 expression during therapy in HCV infected patients compared with healthy controls ..... 203

Figure 4-35; Gating strategy for defining cell differentiation status based on CCR7 & CD45RA expression combined ..... 208
Figure 4-36; Baseline differences and longitudinal changes in CD4+ memory subsets during therapy in HCV infected patients ..... 210
Figure 4-37; Baseline differences and longitudinal changes in CD8+ memory subsets during therapy in HCV infected patients ..... 212
Figure 4-38; Longitudinal changes in CD57 expression during therapy in HCV infected patients compared with healthy controls ..... 213
Figure 5-1; Longitudinal plasma CXCL10 concentrations during PEG-IFN/ribavirin therapy ..... 226
Figure 5-2; Baseline plasma CXCL10 relative to the change in CXCL10 between day 0 and day 1 of PEG-IFN/ribavirin therapy ..... 227
Figure 5-3; Longitudinal plasma CXCL10 concentrations during PEG-IFN/ribavirin therapy ..... 227
Figure 5-4; ΔCXCL10 between day 0 and day 1 of therapy compared with the percentage change in circulating lymphocyte number ..... 228
Figure 5-5; Longitudinal total circulating lymphocyte counts during PEG-IFN/ribavirin therapy ..... 228
Figure 5-6; Correlation between plasma CXCL10 and CXCR3 expression by T cells at baseline ..... 229
Figure 5-7; Correlation between the day 1 change in plasma CXCL10 and expression of CXCR3 by T cells ..... 230
Figure 5-8; Longitudinal plasma CXCL11 concentrations during PEG-IFN/ribavirin therapy ..... 231
Figure 5-9; Correlation between plasma CXCL11 and CXCR3 expression by T cells at baseline ..... 232
Figure 5-10; Relationship between day 1 changes in plasma CXCL10 and CXCR3 expression by CD8+ T cells ..... 232
Figure 5-11; Change in plasma CXCL11 and CXCL10 between day 0 and day 1 ..... 233
Figure 5-12; Plasma CXCL10 concentration stratified by IL28 genotype ..... 234
Figure 5-13; CXCR3-binding chemokine production by Huh7.5 cells following IFN-α stimulation ..... 235
Figure 5-14; FACS plots comparing chemokine receptor expression between IFN stimulated and unstimulated CD3+ lymphocytes ..... 237
Figure 5-15; Changes in CXCR3 expression by IFN-α stimulated T cells ..... 238
Figure 5-16; Changes in CXCR3+ expression by dead PBMCs after in-vitro culture with IFN-α ..... 239
Figure 5-17; In-vitro changes in CXCR3 expression by lymphocytes following IFN-α stimulation in the presence of CXCL10 blockade ..... 240
Figure 5-18; Circulating and organ sequestered lymphocyte counts – IFN-α and control groups compared ..... 242
Figure 5-19; Proportions of circulating, and organ sequestered lymphocytes comprised of CD4+ T cells and CD8+ T cells – IFN-α and control groups compared ..... 243
Figure 5-20; CD3+ lymphocytes as proportions of total lymphocytes isolated from ..... 244
Figure 5-21; Example FACS plots demonstrating CXCR3 expression by circulating and organ sequestered CD8+ T cells ..... 245
Figure 5-22; CXCR3 expression by circulating murine CD8+ T cells – IFN-α and control groups compared ..... 246
Figure 5-23; CXCR3 expression by murine CD8+ T cells isolated from liver, pre-auricular lymph nodes and spleen ..... 246
Figure 5-24; CXCR3 expression by circulating CD4+ T cells in mice ..... 247
Figure 5-25; CXCR3 expression by CD4+ and CD8+ T cells isolated from mouse organs. IFN-α and control groups compared ..... 248
Figure 5-26; CCR6 expression by circulating murine CD8+ T cells ..... 249
Figure 5-27; CCR6 expression by lymphocytes isolated from mouse organs – ..... 250
Figure 5-28; CCR9 expression by circulating murine CD8+ T cells – IFN-α and control groups compared ..... 252
Figure 5-29; CCR9 expression by lymphocytes isolated from mouse organs – ..... 253
Figure 5-30; B7integrin expression on circulating murine CD8+ T cells – ..... 254
Figure 5-31; B7integrin expression by T cells extracted from mouse organs – ..... 255
Figure 5-32; FACS plots demonstrating CCR9/B7integrin expression on CXCR3+ CD8+ T cells ..... 256
Figure 5-33; CXCR3 expression on CCR9+/B7+ CD8+ T cell subsets ..... 257
Figure 5-34; The change in circulating CCR9-/B7integrin- CD8+ T cells – ..... 258

Figure 5-35 FACS plots demonstrating the decrease in B7integrin$^-$/CCR9$^-$ CD8$^+$ T cells (together with decreases in CXCR3 expression) across time in an individual mouse receiving IFN-α ..... 258
Figure 5-36 Organ sequestered CCR9$^-$/B7integrin$^-$ CD8$^+$ T cells – ..... 259
Figure 5-37; B7integrin$^-$, CCR9$^-$ CD8$^+$ T cell populations and their respective CXCR3 expression – mice and humans compared ..... 260
Figure 5-38; FACS plots demonstrating CXCR3 expression on B7integrin$^+$ CD8$^+$ ..... 261
Figure 5-39; Summary of findings related to interaction between exogenous interferon, effector cells and CXCR3 expressing lymphocytes ..... 262

## LIST OF TABLES

Table 1-1 Common abbreviations for characterising HCV infection treatment responses ..... 23
Table 1-2; Recombinant protein vaccine studies ..... 48
Table 1-3; Peptide HCV vaccine studies ..... 51
Table 1-4; DNA vaccine studies* ..... 54
Table 1-5; Viral vectored HCV vaccine studies ..... 56
Table 1-6 Summary of vaccine approaches ..... 57
Table 2-1; Inclusion criteria for HCV003 trial ..... 70
Table 2-2; Exclusion criteria for HCV003 trial ..... 71
Table 2-3; HCV003 Trial arms ..... 72
Table 2-4; Primer combinations and PCR conditions used to sequence autologous virus at relevant epitopes identified in HCV003 trial ..... 81
Table 2-5; FACS panels for THIRST trial ..... 85
Table 2-6; FACS panels for HLA*0201 matched pentamer reactive T cells in HCV003 trial ..... 86
Table 2-7; FACS panel for murine experiments ..... 86
Table 3-1; Demographics of HCV infected patients enrolled in HCV003 trial ..... 102
Table 3-2; Peptides to which T cell IFN-γ ELISPOT responses were detectable prior to vaccination ..... 107
Table 3-3; IFN-γ ELISpot T cell responses at baseline. Circulating viral sequence vs. vaccine insert at immunogenic epitopes ..... 108
Table 3-4; Fine mapping of positive peptide pools identified 3 optimal epitopes ..... 120
Table 3-5; Vaccine induced responses - Circulating viral sequence vs. vaccine insert ..... 125
Table 4-1; THIRST trial baseline patient demographics ..... 149
Table 4-2; Circulating lymphocyte counts during PEG-IFN therapy ..... 151
Table 4-3; Healthy control demographics for volunteers enrolled in the THIRST study ..... 154
Table 4-4; Summary of significant baseline differences and longitudinal changes in lymphocyte subsets during HCV therapy ..... 166
Table 4-5; Chemokine receptor expression by circulating lymphocytes - significant baseline differences and longitudinal changes during therapy in HCV infected patients ..... 186
Table 4-6; Significant baseline differences and longitudinal changes in circulating lymphocyte cytokine profiles during HCV therapy ..... 195
Table 4-7; Significant baseline differences and longitudinal changes in circulating lymphocyte activation during HCV therapy ..... 204
Table 4-8; Significant baseline differences and longitudinal changes in the differentiation status of circulating lymphocytes during HCV therapy ..... 215
Table 4-9; Significant baseline differences and longitudinal changes in circulating lymphocytes during HCV therapy ..... 222
Table 5-1; Percentages of CD4$^+$ and CD8$^+$ T cells expressing CXCR3 ..... 247
Table 5-2; CCR9 expression by CD8$^+$ and CD4$^+$ T cells ..... 251
Table 5-3; Summary of in-vivo IFN-α induced changes in chemokine receptor expression by murine lymphocytes ..... 261

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Amino acid |
| Ab | Antibody |
| Ad | Human Adenovirus |
| Ad6 | Human Adenovirus serotype 6 |
| ChAd3 | Chimpanzee Adenovirus serotype 3 |
| ChAd3-NSmut | Replication defective Chimpanzee Adenovirus encoding the Non Structural region of HCV with mutation in the NS5b catalytic site |
| Ad6-NSmut | Replication defective human Adenovirus encoding the Non Structural region of HCV with mutation in the NS5b catalytic site |
| AE | Adverse event |
| ALT | Alanine aminotransferase |
| APC | Antigen presenting cell |
| BFA | Brefeldin A |
| BSA | Bovine serum albumin |
| CCL | CC-Chemokine receptor |
| CD | Cluster of differentiation |
| CHC | Chronic Hepatitis C |
| ConA | Concavalin A |
| CXCL | CXC-chemokine ligand |
| CXCR | CXC-chemokine receptor |
| DC | Dendritic cell |
| dH20 | Distilled water |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylediaminetetraacetic acid |
| ELISpot | Enzyme-linked immunorbent spot |
| FACS | Fluorescence-activated cell sorter |
| FBC | Full blood count |
| FCS | Foetal calf serum |
| FOXP3 | Forkhead box P3 protein |
| GP | General Practitioner |
| HBV | Hepatitis B Virus |
| HCV | Hepatitis C Virus |
| HIV | Human Immunodeficiency Virus |
| HLA | Human Leukocyte Antigen |
| HRP | Horseradish peroxidase |
| ICS | Intracellular cytokine staining |
| IFN-α | Interferon alpha |
| IFNAR | Interferon alpha receptor |
| IFN/Rib | Interferon and ribavirin |
| IM | Intramuscular |
| CXCL10 | Interferon gamma induced protein-10 |
| CXCL11 | Interferon induced T cell alpha chemoattractant |
| IVDU | Intravenous drug user |
| JRH | John Radcliffe Hospital |
| LDL | Low density lipoprotein |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| MHC | Major histocompatibility complex |
| MIG-I/CXCL9 | Monokine induced by gamma interferon |
| NK | Natural killer cell |
| NS | Non structural |
| MAVS | Mitochondrial antiviral signaling protein |
| MVA | Modified Vaccinia Ankara |
| MSM | Men who have sex with men |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphatase buffer saline |
| PCR | Polymerase chain reaction |
| PEG-IFN | Pegylated interferon-alpha |
| PKR | Protein kinase R |
| PMA | Phorbal 12-myristate 13-acetate |
| qPCR | Quantitative real time polymerase chain reaction |
| RBV | Ribavirin |
| RNA | Ribonucleic acid |
| RPM | Revolutions per minute |
| SAE | Serious adverse event |
| SEM | Standard Error of Mean |
| SFU | Spot forming units |
| SC | Subcutaneously |
| SRB1 | Scavenger receptor class B-1 |
| STAT | Signal transducer and activator of transcription protein |
| SUSAR | Suspected Unexpected Serious Adverse Reaction |
| SVR | Sustained virological response |
| TNF-α | Tumour necrosis factor - alpha |
| Treg | T regulatory cell |
| VP | Virus Particles |

## STANDARD INTERNATIONAL UNITS AND THEIR PREFIXES

| Quantity | Unit | Abbreviation |
|---|---|---|
| Mass | kilogram* | kg |
| Length | meter | m |
| Time | second | s |
| Quantity of substance | mole | mol |
| Temperature | kelvin | K |
| Electrical current | ampere | A |
| Luminosity | candle | cd |
| **SI unit prefixes** | **Factor** | **Symbol** |
| Peta | $10^{15}$ | P |
| Tera | $10^{12}$ | T |
| Giga | $10^{9}$ | G |
| Mega | $10^{6}$ | M |
| Kilo | $10^{3}$ | k |
| centi | 0.01 | c |
| milli | $10^{-3}$ | m |
| micro | $10^{-6}$ | µ |
| nano | $10^{-9}$ | n |
| pico | $10^{-12}$ | p |
| femto | $10^{-15}$ | f |
| atto | $10^{-18}$ | a |
| Compiled from *http://www1.bipm.org/en/si/*<br>*A tolerated anomaly: the basic unit of mass *(kg)* has a prefix | | |

## DERIVED UNITS WITH ASSIGNED NAMES THAT ARE USED IN THIS THESIS

| Quantity | Unit | Abbreviation |
|---|---|---|
| Electromagnetism (potential) | volt | V |
| Temperature | celsius | °C |
| Volume | litre | l |
| Pressure | pascal | pa |

# Chapter 1 Introduction

## 1.1 HEPATITIS C INFECTION

Hepatitis C virus (HCV) infects more than 170 million people globally with another 3 million people newly infected each year (WHO 1997; Beuers et al. 2009). Following acute infection, 15-20% of those infected eradicate virus over weeks or months and are often asymptomatic whilst the remaining 80% of people will develop chronic disease (Allison et al. 2012). Approximately 20% of patients with chronic HCV infection eventually develop liver cirrhosis and 1 - 5% develop liver cancer (Afdhal 2004; Lauer & B. D. Walker 2001; Tong et al. 1995). More recent work has suggested lower rates of disease progression with only 2% of HCV infected patients developing cirrhosis after a mean duration of infection of 25 years (Allison et al. 2012). Even with these low rates of disease progression, HCV infection is now the leading cause of liver transplantation in many western countries (NDDIC 2010).

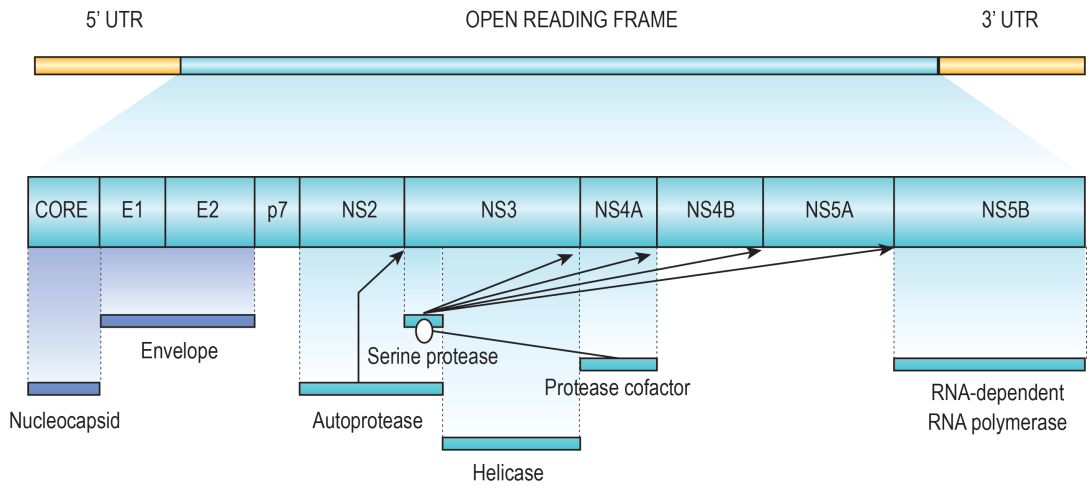
Disease prevalence varies by region, with the highest rates in parts of Asia and Africa where up to 20% of the population are infected (Alter 2007). Current treatment for HCV infection is pegylated interferon and ribavirin, with the addition of a protease inhibitor (boceprevir or telaprevir) in those with genotype 1 infection. Treatment is expensive, prolonged, has an extensive side effect profile and frequently fails. Clearly then there is a real need for both a prophylactic vaccine that will prevent or attenuate primary infection, and also a potential role for a therapeutic HCV vaccine that will increase cure rates of infected patients. Substantial progress has been made in this endeavor in recent years.

### 1.1.1 AETIOLOGY

HCV is a particularly fastidious virus and has only been shown reproducibly to replicate in the hepatocytes of humans and chimpanzees. The virus is a single stranded RNA virus with an enveloped virion belonging to the family Flaviviridae (Moradpour et al. 2007).

The positive sense RNA genome is approximately 9600 nucleotides in length. A single HCV polyprotein comprised of 3011 amino acids is translated from the genome and subsequently cleaved by cellular and viral proteases into 3 structural proteins (core, E1 and E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Grakoui et al. 1993; Reed & Rice 2000) (Figure 1-1).

Figure 1-1; The HCV genome and expressed polyprotein

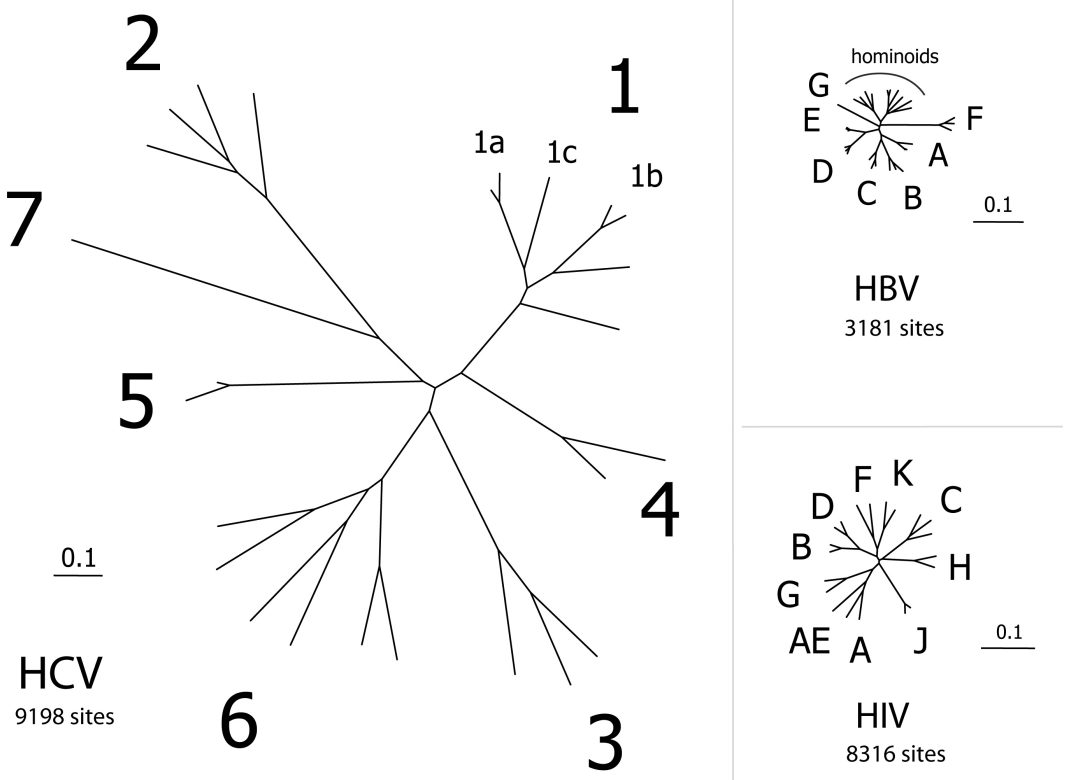



The virus consists of a single open reading frame and 2 untranslated regions (UTRs). It encodes a polyprotein of approximately 3000 amino acids, which is cleaved into single proteins by a host signal peptidase in the structural region and the HCV-encoded proteases in the nonstructural (NS) region. The structural region contains the core protein and 2 envelope proteins (E1 and E2). 2 regions in E2, called hypervariable regions 1 and 2 (HVR 1 and HVR 2), show extreme sequence variability. E2 also contains the binding site for CD81, the putative HCV receptor or coreceptor. The nonstructural proteins have been assigned functions as proteases (in the case of NS2, NS3, and NS4A), helicase (in the case of NS3), and RNA-dependent RNA polymerase (NS5B). Although the crystal structure of NS3 and NS5 is known[1] the function and properties of the other proteins (such as p7) are less well characterised. Adapted from Lauer & B. D. Walker 2001

The envelope proteins mediate viral cell entry by binding to a number of cell surface receptors (e.g. LDL receptor, CD81, SRB1 and claudin) (Flint et al. 2001).

HCV mutates at nearly one nucleotide per replication cycle as a consequence of the poor fidelity of its NS5B viral polymerase (which lacks proofreading function) (Bukh et al. 1995). This, in conjunction with a short viral half-life and rapid turnover (just a few hours), results in a high genetic variability. Two regions in E2, called hypervariable regions 1 and 2 (HVR 1 and HVR 2) are the most variable - this is thought to be the result of selective pressure by virus-specific antibodies. As a consequence of this high genetic variability, many distinct but closely related HCV variants, known as quasispecies are typically found in each infected individual (Bukh et al. 1995). It is estimated that HCV viral diversity is 10 times greater than that found in human immunodeficiency virus (HIV) infection (Figure 1-2) and this clearly represents a significant challenge to successful vaccine development; an immune response that protects against infection by one genotype or quasispecies may not protect against another.

Figure 1-2; Comparison of the phylogenetic trees of HCV, HIV and Hepatitis B virus




*Adapted from Ray & Thomas 2009. The HCV phylogenetic tree demonstrates the 7 branches specific for each genotype and the marked genetic diversity of this virus compared with the left 2 phylogenetic trees for HIV and hepatitis B virus (HBV). Hominoids = non-human (orang-utans,chimpanzees and gorillas)

Studies based on molecular evolution of African and Asian Hepatitis C viruses suggest that HCV first appeared over 1000 years ago (Simmonds 2001). Subsequently, HCV evolved in discrete geographical regions giving rise to 7 major genotypes (1 - 7). These genotypes share a genetic homology of approximately 80% and are further subdivided into more than 100 subtypes (Simmonds et al. 2005; Cooper et al. 1999). In recent decades there has been a global epidemic of a few subtypes (1a, 1b, 3a, 2a and 4a) associated with intravenous drug use and modern medical practice. HCV genotype not only has relevance in relation to geographic distribution but more importantly in determining response to current medical therapy (Grieve et al. 2006).

### 1.1.2 CLINICAL FEATURES AND DIAGNOSIS OF HCV INFECTION

HCV is transmitted largely through blood or blood-contaminated products. In the West prior to 1990, the most common method of HCV transmission was through blood transfusion. Following the introduction of routine blood screening in 1990, the risk of transmission from a blood transfusion fell to about 1 in 2 million (Stramer et al. 2004). However, in developing countries, reuse of poorly disinfected needles in health care, and blood transfusions from unscreened donors remain significant and substantial causes of HCV transmission.

In the West, HCV is now most commonly transmitted through the sharing of contaminated needles by intravenous drug users (IVDUs), and this accounts for about 70 – 90% of new infections (Lauer & B. D. Walker 2001, HPA 2011) Amongst the HIV infected population, the incidence of HCV infection in men who have sex with men has risen sharply in recent years and is now higher in this group than it is for IVDUs (4.1 per 100 person years vs. 2.1 per 100 person years, respectively) (Wandeler et al. 2012). This is not true (in the UK at least) for non-HIV infected individuals where the incidence of HCV infection in men who have sex with men has actually declined in recent years (HPA 2011). The remainder of new HCV infections result from occupational exposure (such as a medical needlestick injury) and rarely perinatal transmission or heterosexual intercourse (Terrault et al. 2012). In about 20% of cases, the source of HCV infection is never identified (Wasley et al. 2007).

Primary (acute) infection with HCV is usually asymptomatic, though sometimes patients may present with jaundice. Considering the asymptomatic nature of primary infection, it is likely that the true prevalence of infection is greater than reported. Following acute infection approximately 20% of patients will spontaneously clear virus, whilst 80% will

develop persistent disease (Afdhal 2004; Allison et al. 2012). Persistent HCV is a significant cause of patient morbidity and mortality as patients develop progressive liver fibrosis. Early studies suggested that 20 - 50% of patients eventually develop cirrhosis and 1 - 5% develop hepatocellular cancer (Lauer & B. D. Walker 2001). In a more recently published study, only 2% of 469 blood donors who were positive for HCV developed cirrhosis after a mean duration of infection of 25 years. Nevertheless, HCV infection is now the most common cause of liver transplantation in the USA, and has risen as the indication for liver transplantation from 10% to 18% of cases in England between 1996 and 2010 (NDDIC 2010; HPA 2011).

There are 2 broad categories of diagnostic tests for HCV infection; i) serology tests that detect HCV antibodies, and ii) molecular assays that detect or quantify HCV RNA;

i) HCV screening is typically performed by enzyme-linked immunoassay (or chemoluminescence assay) on patient serum/plasma. These tests all employ HCV-encoded recombinant proteins to detect HCV antibodies and have a specificity of >99%. However, even a specificity of 99% does not provide the desired positive predictive value – in populations with a low HCV prevalence (< 10%), the false positive rate ranges between 15 - 60% (Alter, Kuhnert, and Finelli 2002). For this reason, positive screening tests are confirmed either by PCR or strip immunoblot assay (RIBA). The strip immunoblot assay (Chiron RIBA® HCV 3.0 SIA) uses recombinant HCV antigens and synthetic peptides to detect HCV antibodies in patient serum/plasma.

ii) HCV RNA tests are used to confirm screening tests, determine HCV genotype and quantify viral load. They are also recommended for HCV screening in patients in whom serology sensitivity may be impaired such as the immunocompromised.

### 1.1.3 CURRENT MEDICAL THERAPY FOR HCV (INCLUDING MECHANISMS OF ACTION)

Current gold-standard therapy for HCV infection is weekly subcutaneous injections of pegylated interferon-alpha (PEG-IFN) combined with daily oral ribavirin for a period of 24 weeks for genotypes 2 and 3, and 48 weeks for genotypes 1 and 4. Recently 2 viral protease inhibitors (telaprevir and boceprevir) have also been approved for use in genotype 1 HCV infection. These need to be used in combination with PEG-IFN/ribavirin therapy to avoid the rapid emergence of viral mutants.

Current therapy is fraught with significant side effects and leads to a sustained virological response (SVR; see Table 1-1) in approximately 40 to 50% of patients with genotype 1 infection, 65 to 70% with genotype 3, and 80% of those with genotype 2. It is expected the recent addition of protease inhibitors for genotype 1 infection will increase SVR rates to 70% but 'real life' data relating to their use outside the trial setting is not yet available (Kwo et al. 2010; Marcellin et al. 2010).

| Table 1-1 Common abbreviations for characterising HCV infection treatment responses | |
|---|---|
| RVR; rapid virological response | HCV undetectable **4 weeks** after commencing standard HCV therapy* |
| Partial EVR; partial early virological response | > 2 $\log_{10}$ drop in circulating viral load from baseline **12 weeks** after commencing standard HCV therapy* |
| EVR; early virological response | HCV undetectable **12 weeks** after commencing standard HCV therapy* |
| SVR; sustained virological response | HCV undetectable **6 months** after completing standard HCV therapy* |
| *measured by quantitative PCR on patient plasma | |

The cost of current HCV therapy is substantial. PEG-IFN and ribavirin treatment costs an average of approximately £7000 in the United Kingdom for a treatment course (Grieve et al. 2006), and the cost of adding a protease inhibitor in the UK is ranges between £9,000 - 15,000 (personal correspondence, Dr Jane Collier, Hepatologist, John Radcliffe Hospital, Oxford). Clearly these costs put such therapy out of the reach of many, particularly in the developing world.

Many promising direct antiviral therapies for chronic HCV infection are in development. However, it is unlikely any of these will reach clinical application in the next few years, and, like the new protease inhibitors, their cost will be significant. The NS5B polymerase inhibitor GS-7977 is one such therapy that recently entered a phase III clinical trial (Clinical trial ID: NCT01625338). There are however, concerns for this drug class as a whole following the recent report of congestive cardiac failure in a patient being treated with another polymerase inhibitor, BMS-986094. Similarly, recent reports of fatal pancreatitis with the cyclophilin inhibitor, Alisporovir have raised concerns for this therapeutic drug class (http://www.fiercebiotech.com/tags/alisporivir).

Interestingly, the SVR rate of individuals that are treated in the early phase of infection with interferon alone is very high (70 - 90%) compared with treatment results once chronic infection is established (Wiegand et al. 2006). The reasons for this are not known but may relate to the fact that the infecting virus has not yet evolved to subvert the effects of PEG-IFN, or alternatively, PEG-IFN therapy may harness the host's robust natural immunity that is present in acute infection in a way that it is unable to do so once chronic infection is established (Lauer et al. 2005). Similarly, it is plausible that a therapeutic vaccination strategy may prove more efficacious in early infection. In clinical practice however, acute infection is frequently asymptomatic so that patients usually do not present to clinicians until chronic infection is established.

Clearly then, a therapeutic vaccine that increased SVR rates in chronic infection, or reduced the duration of standard therapy would represent a major step forward.

## *INTERFERON-α*

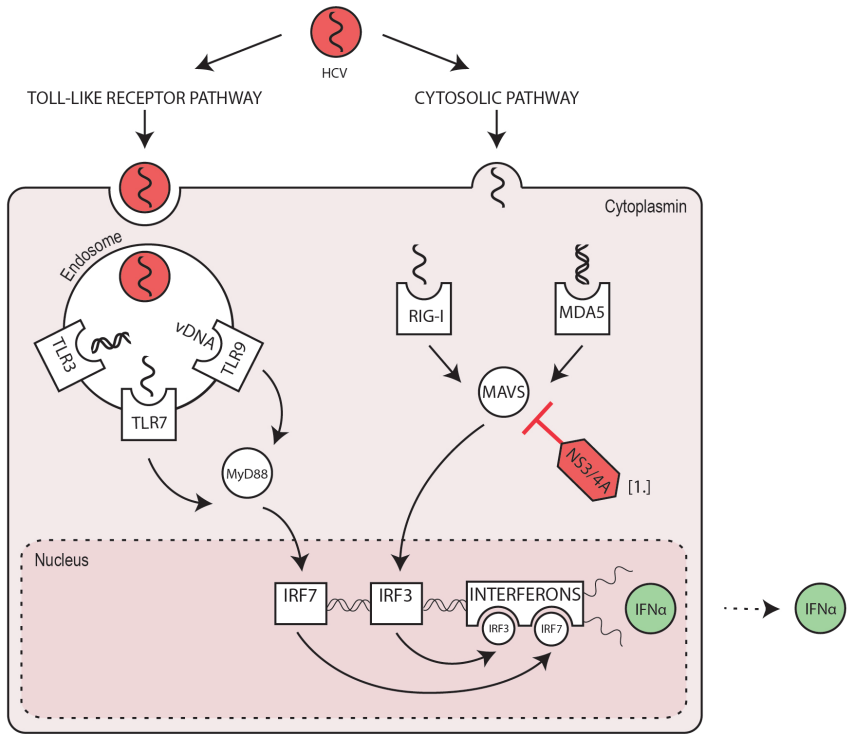
The interferon system forms part of the body's first line of defense against viral infection. These cytokines are induced in mammalian cells and secreted in response to viral infection to activate a global anti-viral state. For many years after their original discovery, research focused on the direct antiviral and anti-proliferative effects of the interferon family (Belardelli & Gresser 1996). More recently, their pleiotropic and profound effects on cell physiology, and particularly the critical role of interferons in forming a link between innate and adaptive immunity has been recognised (Belardelli & Ferrantini 2002).

Together interferon-α (13 subtypes) and interferon-β (2 subtypes) make up the type I interferon family, so called because members of this cytokine family bind type 1-interferon receptors (IFNARs). Each subtype is encoded by its own gene (clustered on chromosome 9), is regulated by its own promoter, and differs substantially in its specific antiviral activities. The molecular basis for these differences is not yet known (Heim 2012).

## Endogenous production of type 1 interferons

Type I interferons are produced by cells in response to viral infection through 2 key pathways that detect viral genomes: the toll-like receptor (TLR) dependent pathway (Iwasaki & Medzhitov 2004) and the cytosolic pathway (Yoneyama & Fujita 2007). These pathways are outlined in Figure 1-3. The initial production of interferon-α (IFN-α) results in a strong positive feedback loop that leads to very high local concentrations of IFN-α (Marié et al. 1998).

Figure 1-3 HCV mediated stimulation of endogenous IFN-α production



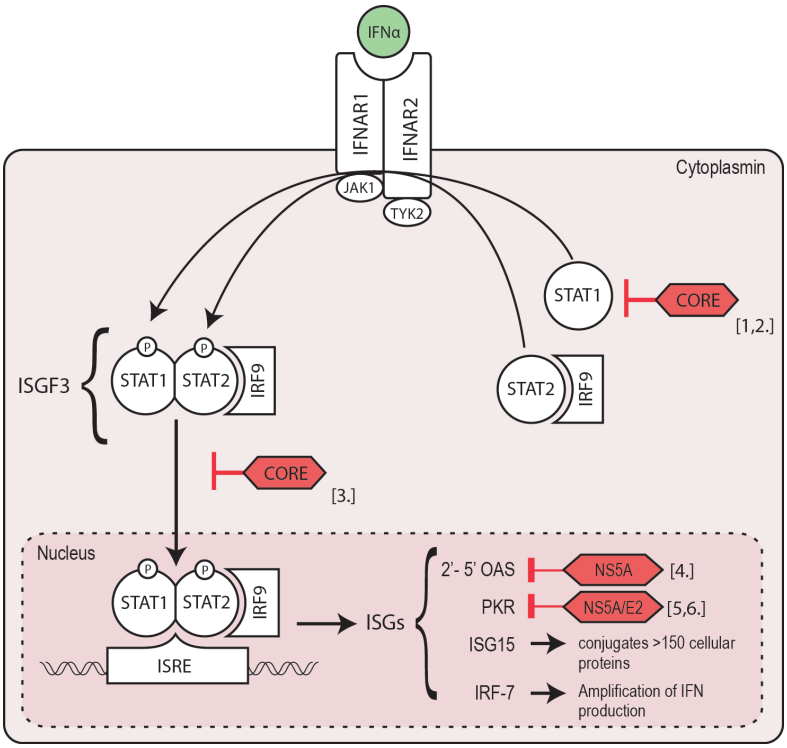

HCV infection stimulates production of endogenous IFN-α through 2 distinct pathways;
i) The toll-like receptor (TLR) dependent pathway: TLRs are a family of pattern recognition receptors that recognise microbial pathogens and are typically expressed by antigen presenting cells. TLR3, TLR7 and TLR9 are localized to intracellular compartments (such as endosomes) and detect double stranded RNA (dsRNA), single stranded RNA (ssRNA) and unmethlyated DNA respectively. Once activated, a signaling cascade follows that leads to production of interferon regulatory factors (IRF), particularly IRF7 through activation of MyD88, and ultimately production of type I interferons.

ii) The cytosolic pathway: ssRNA, and dsRNA bind to RNA helicases retinoic acid inducible gene-1 (RIG-I) and melanoma differentiation antigen 5 (MDA5) respectively. This leads to binding of MAVS, essential downstream adaptor in the cytosolic pathway. Through a signaling cascade MAVS activate transcription of interferon regulatory factor 3 (IRF3) to promote production of type I interferons.
[1] Li et al. 2005

## Downstream effects of IFN-α

IFN-α binds to specific cell surface receptors activating the Jak-STAT signal transduction pathway, and this in turn leads to transcription of hundreds of IFN-stimulated genes (ISGs). The IFN-α signal transduction pathway is outlined in Figure 1-4, together with the mechanisms through which HCV subverts the normal IFN-α response (for an excellent review – Heim et al. 2012). Only a few of the protein products of this process are well characterised and many of these genes remain the subject of intense research today.

Figure 1-4 The downstream effects of IFN-α, together with mechanisms through which HCV subverts these responses




IFN-α binds to the IFNAR1/IFNAR 2 receptor complex to activate the receptor associated tyrosine kinases Jak1 and Tyk2. The kinases then phosphorylate and activate STAT1 and STAT2, and combine with a third transcription factor IRF9, to form interferon-stimulated gene factor 3 (ISGF3). This translocates to the nucleus where it binds specific DNA elements (ISRE: Interferon stimulated regulatory element) to promote transcription of hundreds of ISGs (interferon stimulated genes). HCV core protein (core) impairs STAT1 activation and nuclear translocation of ISGF3. HCVNS5A inactivates 2'-5' oligoadenylate synthetase (OAS), HCV NS5A and envelope protein (E2) have been shown to inactivate PKR.
[1] Lin et al. 2005, [2] Bode et al. 2003, [3] Melen et al. 2004, [4] Taguchi et al. 2005, [5] Gale et al. 1997 and [6] Taylor et al. 2001

The anti-viral effects of IFN-α broadly stem from 2 distinct but complementary mechanisms:

i) **Induction of a non-virus-specific antiviral state in infected cells resulting in inhibition of viral replication** (Sen 2001; Schoggins et al. 2011).

Examples include:

a. Activation of protein kinase R (PKR) – a member of the eukaryotic initiation factor kinase family, which blocks translocation of most cellular and viral mRNAs (Sadler and Williams 2008).

b. Increased expression of 2' - 5' oligoadenylate synthetase (OAS) – activates the latent host RNA nuclease RNase L, causing degradation of viral (and host) RNAs (Sadler and Williams 2008).

c. Interferon stimulated gene-15 (ISG15) – is a ubiquitin like protein that is upregulated by type I IFNs and conjugates more than 150 cellular target proteins (Zhao et al. 2005).

**ii) Immunomodulatory effects that enhance the host's innate and specific antiviral immune responses and accelerate the death of infected cells.**

Examples include:

a. Induction of dendritic cells to proliferate and differentiate leading to better priming of naïve T cells (Le Bon et al. 2001).

b. MHC class I up-regulation in tumor cells and other targets such as hepatocytes (Johnson & Pober 1990; Clayton et al. 2005).

c. Activation and proliferation of NK cells and macrophages (Krug et al. 2004; Randall & Goodbourn 2008)

d. Influencing lymphocyte trafficking to areas of inflammation through production of chemokines such as Interferon gamma induced protein 10

(CXCL10) (Padovan et al. 2002).

**HCV interference with the host IFN-α response**

Not surprisingly, at every level viruses have evolved mechanisms that interfere with the normal functioning of the IFN-α system (Sen 2001). HCV subverts this system through a number of mechanisms (illustrated in Figure 1-3 and Figure 1-4) that can be grouped under 2 broad headings:

**i) Inhibition of IFN-α production**

a. The NS3/4A HCV protease cleaves and inactivates mitochondrial antiviral signaling protein (MAVS), an innate immune signaling protein that is important for IFN-α production (Li et al. 2005).
b. Inhibiting the downstream effects of IFN-α (described below) indirectly inhibits IFN-α production by impairing the positive feedback loop.

**ii) Inhibition of the downstream effects of IFN-α**

a. HCV core protein; i) induces degradation of STAT1 (Lin et al. 2005), or inhibits STAT activation (Bode et al. 2003), and ii) impairs nuclear translocation of the ISGF3 complex (Melen et al. 2004).
b. HCV NS5A protein; i) inactivates interferon-induced protein kinase (PKR) (Gale et al. 1997), and ii) may interfere with 2'-5' oligoadenylate synthetase (OAS) activity (Taguchi 2004).
c. Envelope protein (E2) may inactivate interferon-induced protein kinase (PKR) (Taylor et al. 2001).

## *RIBAVIRIN*

Ribavirin is an oral purine nucleoside analogue with broad anti-viral activity (Shigeta et al. 1992; Hosoya et al. 1993). The key mechanism of action of ribavirin in CHC remains controversial and multiple alternatives have been suggested. Like IFN-α these can be broadly grouped under 2 headings; i) direct impairment of viral replication, and ii) improvement of the host immune response.

### i) Direct impairment of viral replication

As a nucleoside analogue, incorporation of ribavirin into the viral genome can lead to mutagenesis. This in turn may lead to 'error catastrophe', so called because inappropriate nucleoside pairing blocks viral replication, in the case of HCV by impairing HCV-RNA dependent RNA polymerase (NS5B) function (Cameron & Castro 2001). Work by Young and colleagues has however, indicated that although ribavirin therapy is indeed associated with early, transient increases in HCV mutation rates, lethal mutagenesis and error catastrophe is unlikely to explain its mechanism of action (Young et al. 2003). Indeed, in this study a specific NS5B mutation developed in all patients during ribavirin monotherapy that restored viral replication.

### i) Improving host immune responses

When used as monotherapy for CHC, ribavirin has minimal effect on viremia but transiently normalises ALT levels in most patients (Reichard et al. 1993). However, when combined with IFN-α, ribavirin clearly has a synergistic anti-HCV effect. These observations led researchers to investigate the possibility that ribavirin may enhance host immune responses to CHC. Ribavirin has been shown to exert a number of influences in a range of small studies that have not been reproduced:

- In vivo, ribavirin has been shown to reduce immune complex formation and thereby modulate humoral immune responses. Additionally, based on a study comparing patients on combined therapy with IFN-α monotherapy, ribavirin may increase serum IL-18 (Murata et al. 2005) which is an important pro-inflammatory cytokine.

- Ex vivo/in vitro, ribavirin has been shown to reduce expression of IFN-γ, TNF-α, IL-10 and IL-12 by dendritic cells, as well as decreasing DNA, RNA and protein synthesis (Barnes et al. 2004). Ribavirin may also increase Th1 responses in patients with chronic HCV infection (Shiina et al. 2004).

- Based on microarray data, ribavirin enhances interferon stimulated gene (ISG) responses and inhibits production of chemokines MIP1-α and MIP1-β (Y. Zhang et al. 2003).

### *DIRECT ANTI-VIRAL AGENTS – BOCEPREVIR AND TELAPREVIR*

Two protease inhibitors, boceprevir and telaprevir have recently been approved for treatment of chronic genotype 1 HCV infection. Both act by inhibiting viral NS3/4A protease function, which is necessary for proteolytic cleavage of the HCV polyprotein during viral replication (Alkhouri & Zein 2012). As outlined in Figure 1-3, the viral NS3/4A protease also cleaves host cell MAVS thereby impairing endogenous IFN-α production in response to viral infection. Therefore, although evidence to support it is not yet available, NS3/4A protease inhibitors may help stimulate the innate immune response to HCV infection by restoring interferon signaling.

## 1.2 HOST IMMUNE RESPONSES TO HEPATITIS C INFECTION

### 1.2.1 RELEVANCE TO VACCINE DEVELOPMENT

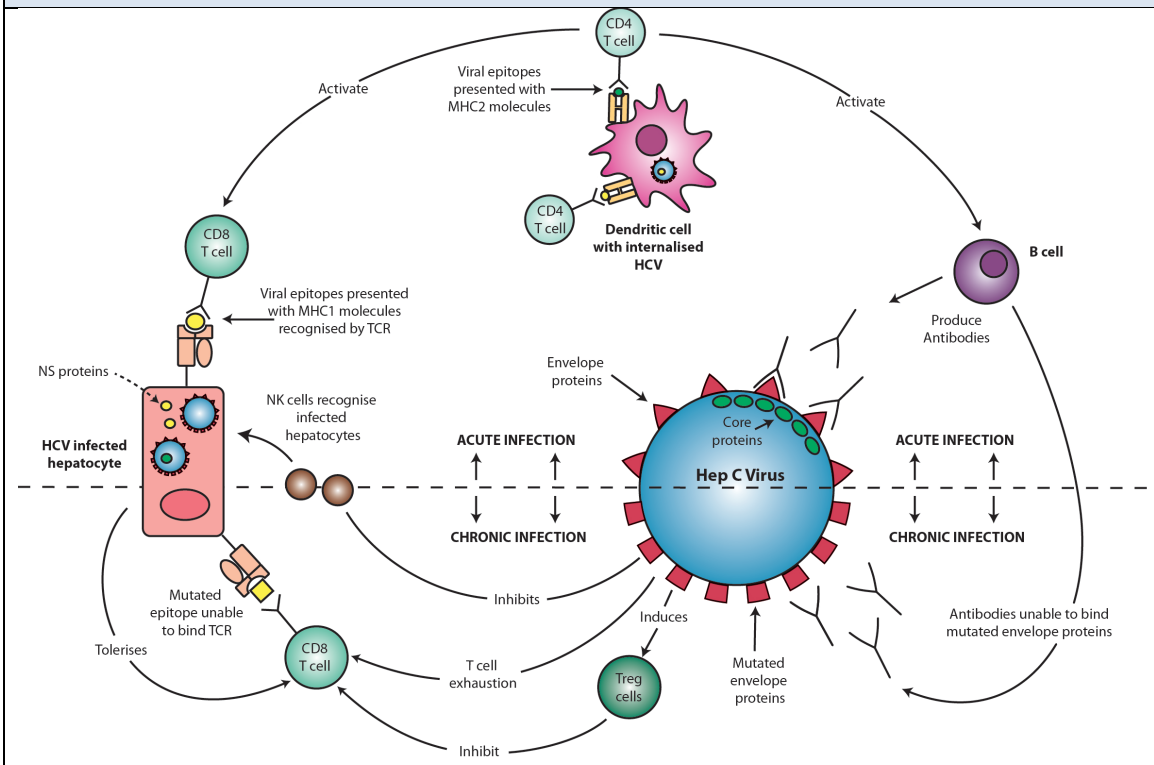
Following acute infection with HCV, approximately 20% of people will spontaneously clear virus (Afdhal 2004). This is in stark contrast to Human Immunodeficiency Virus (HIV) where infection inevitably persists. The distinct clinical outcomes that follow acute HCV infection allow comparative analysis of anti-viral immunity between these clinical groups, and this has been the major focus of many research groups over the last decade.

Although the exact mechanisms behind successful viral clearance are not yet fully elucidated, it appears that multiple components of the immune system, both innate and adaptive, play a crucial role in this process (Figure 1-5). The recent discovery that polymorphisms in the IL-28 gene are important in determining outcomes, both of acute infection and HCV treatment, has highlighted the important role of innate immunity in HCV infection (Ge et al. 2009; Thomas et al. 2009; Tanaka et al. 2009). Another key observation is that strong, broad adaptive immune responses are detected during acute infection and these persist in those who resolve infection – whereas persistent infection is associated with a weak, frequently undetectable HCV specific T cell response (Wertheimer et al. 2003; Grakoui et al. 2003; Diepolder et al. 1995; Thimme et al. 2001; Wiesch et al. 2005).

Is there any evidence then that the induction of adaptive immune responses during acute-resolved infection protects the host from subsequent viral challenge? If so, then a HCV vaccine that mimics these responses might well afford protection. Work in the chimpanzee model has suggested this might not be the case – chimpanzees that have recovered from HCV infection can be re-infected with the same inoculum (Farci et al. 1992; Prince et al.

1992; Bukh et al. 2008). However, there is evidence that both chimpanzees and humans who have previously cleared HCV are at least partially protected against re-infection (Osburn et al. 2010; Bassett et al. 2001; Mehta et al. 2002; Nascimbeni et al. 2003). Rather than preventing acute infection with sterilising immunity, typically this form of protective immunity works by preventing progression to chronic infection following repeat HCV exposure. These findings suggest that a vaccine that induces and exploits similar protective responses may ultimately succeed in preventing chronic HCV infection. Since chronic infection, and not acute infection is associated with significant morbidity and mortality, prevention of chronicity is an acceptable end-point.

Figure 1-5; Schematic representation of viral/ host immune interactions during acute and chronic HCV infection




In acute HCV infection (above horizontal dotted line), both the adaptive and innate immune response are engaged to help clear infection. Priming of antigen specific T cells by dendritic cells permits $CD8^+$ T cells to recognise and target viral antigen presented in MHC class I molecules by infected hepatocytes. HCV specific $CD4^+$ T cells recognise viral antigen presented in MHC class II molecules and augment the $CD8^+$ T cell response together with humoral immune responses. B cells produce neutralising antibodies that bind extra-cellular viral components such as envelope proteins. In chronic infection multiple mechanisms contribute to the failure of viral clearance. HCV specific $CD8^+$ T cell function is impaired through i) Treg induced suppression, ii) exhaustion due to chronic antigen exposure, iii) viral escape, and iv) the 'tolerising' environment of the liver. Viral escape also permits evasion of neutralising antibodies. NK cell function is directly impaired by viral NS proteins.

## 1.2.2 ANTI-HCV SPECIFIC T CELL RESPONSES

The HCV specific T cell response has been shown to play a crucial role in determining the outcome of primary HCV infection. Firstly, comparative studies in man have demonstrated that a broad and sustained $CD8^+$ and $CD4^+$ T cell response targeting multiple HCV regions is associated with spontaneous viral clearance. Conversely, a weak and narrowly targeted T cell response is a hallmark of persistent infection (Wertheimer et al. 2003; Grakoui et al. 2003; Diepolder et al. 1995; Thimme et al. 2001; Wiesch et al. 2005; Cox et al. 2005). Secondly, immunogenetics studies from single source outbreaks and from mixed

populations have shown a clear association between different HLA class I and II alleles and viral clearance (McKiernan et al. 2004; Neumann-Haefelin & Thimme 2007). Both HLA B27 and HLA A3 were shown to be protective against the development of chronic infection following an outbreak of HCV Genotype 1b infection in Irish women in 1977. Thirdly, evidence from the chimpanzee model, has shown that once protective responses have been induced, depletion of either $CD4^+$ or $CD8^+$ T cells leads to loss of control over repeated HCV challenge (Grakoui et al. 2003; Shoukry et al. 2003).

In comparison to those who clear acute HCV infection, studies examining patients who develop chronic infection suggest that both the quality and quantity of their $CD4^+$ and $CD8^+$ T cell responses are impaired (Lauer et al. 2004; Bowen & C. Walker 2005). It is not yet clear however, to what extent this is a cause or a consequence of chronic infection. Proposed mechanisms by which the T cell response is attenuated include:

i) Viral escape from T cell recognition; T cells recognise short viral peptides (T cell epitopes) bound in the groove of MHC molecules. In the presence of selective pressure driven by T cells, viral variation in T cell epitopes that arise during viral replication may abrogate this recognition – so called 'viral escape'. To varying degrees, viral escape mutations may be associated with reduced viral fitness. T cell escape in HCV infection has clearly been demonstrated such that many T cell responses detected during chronic infection target a 'historical' epitope that is no longer found in circulating virus. However, in many cases T cells clearly do target autologous circulating virus – so therefore, T cell escape cannot fully explain the paucity of responses seen in chronic infection. This also means that therapeutic vaccination may be able to 'rescue' responses that are still able to target the host's circulating virus.

ii) T cell exhaustion; whereby chronic antigen stimulation results in a reduction in antigen specific T cell frequency and function. This concept was first recognised in murine models of chronic viral infection. T cell exhaustion is now thought to play a role in chronic HCV infection (Bengsch et al. 2010; Barber et al. 2005; Gruener et al. 2001; Wedemeyer et al. 2002; Urbani et al. 2002). The mechanisms underlying exhaustion are poorly understood but include expression of inhibitory receptors such as the molecules PD-1 and CTLA-4 that mediate T cell function (Bengsch et al. 2010; Blackburn et al. 2009; Golden-Mason et al. 2007. These receptors are normally involved in self-tolerance, but in the setting of chronic infections may be upregulated, presumably to evade immunopathology. One of the key suggestions from murine models of persistent viral infection, is that repair of these 'defective' T cell responses is most readily achieved in the setting of a lowered viral load (Wherry et al. 2005). Thus, the idea of vaccination or immunomodulation as an adjunct to conventional antiviral therapy has recently emerged.

iii) Regulatory immune cell subsets; Tregs ($FOXP3^+$) that may actively suppress $CD8^+$ and $CD4^+$ T cell responses are increased in chronic HCV infection. Indeed, depletion of Tregs does lead to a consistent boost in T cell function during HCV infection (Rushbrook et al. 2005). This may well be a consequence of chronicity rather than a cause, but nevertheless it might serve to limit the efficacy of immunotherapy unless it is reversed.

iv) The liver itself is thought to represent a tolerogenic environment. In evolutionary terms this would serve to protect the liver that is constantly exposed to antigens via the portal tract, from chronic inflammation. Interestingly, in organ

transplantation, the liver is the only organ where HLA matching between donor and recipient is not required (Poli et al. 1998). This observation raises the intriguing possibility that anti-viral T-cells primed in the periphery by vaccination may induce T-cells that are of a 'superior' quality to those primed in the liver during natural infection.

### 1.2.3 HUMORAL IMMUNITY TO HCV

Circulating antibodies to HCV are usually detectable within 1 to 3 months of HCV infection (Schmilovitz-Weiss et al. 1993) and appear to be an important component to viral control in early infection. There is a direct correlation between viral clearance during acute infection and the rapid induction of a high-titre of circulating cross-neutralising antibodies (Pestka et al. 2007). However, neutralising antibodies are also found in high titres in the majority of chronically infected patients and clearly, in these cases are unable to control infection (Bartosch, Bukh, et al. 2003a).

The envelope protein, which is the major target for HCV antibodies, displays some of the virus' highest levels of genetic heterogeneity. It is likely that variation between quasispecies in these envelope proteins allows the virus to evade host neutralising antibodies (Farci et al. 2000). This contrasts to other viruses such as the genetically stable Hepatitis B virus in which persistent humoral immunity has allowed successful development of a prophylactic vaccine.

There are several other mechanisms by which HCV evades humoral immunity including direct cell to cell viral transfer (Timpe et al. 2007), induction of antibodies that interfere with neutralising antibodies (P. Zhang et al. 2009; Hahn et al. 2007), and the shielding of neutralising epitopes by glycosylation of defined amino acids of envelope glycoproteins

(Helle et al. 2007). For these reasons, it seems unlikely that an antibody-mediated vaccine in isolation will successfully induce sterilising immunity –however, such a strategy may have an important role, either as an adjunct with other approaches, or in attenuating the course of acute infection.

### 1.2.4 INNATE IMMUNITY

The fact that IFN-α forms the mainstay of treatment of HCV clearly demonstrates that innate immune cytokines can eradicate the virus. However, the mechanism of action of interferon is complicated- having both direct anti-viral actions through the inhibition of protein kinase R and cellular protein production, and through diverse effects on immune cellular function. A more detailed description of the mechanisms through which IFN-α exerts its effects is presented in Section 1.1.3 (page 22).

Although early studies suggested that HCV specific T cell responses are enhanced by IFN-α therapy (Kamal et al. 2002; Cramp et al. 2000), more recently, this has been challenged by studies that show that HCV specific T cells and total T cell counts decline in peripheral blood as treatment proceeds (Barnes et al. 2009; Burton et al. 2008). The reasons for this decline are unclear, but if they reflect a true failure of T cell production rather than migration, then therapeutic T cell vaccination strategies will need to overcome this.

Recent genome wide association studies and candidate gene studies have further highlighted the crucial importance of innate immunity during HCV infection. These studies identified a cluster of seven host single nucleotide genetic polymorphisms (SNPs) linked to interferon-λ3 (also known as IL28B), that are important in determining both spontaneous viral clearance during acute infection, and also the response to standard PEG-IFN/ribavirin therapy (Suppiah et al. 2009; Tanaka et al. 2009; Thomas et al. 2009;

Ge et al. 2009)2. Individuals homozygous for the protective IL28 alleles have viral clearance rates following treatment that are approximately 3 to 5 times higher than those of patients who are homozygotes for the risk allele (Thomas et al. 2009; Rauch et al. 2010).

The observations related to IL28 polymorphisms have created great interest in the field, and the biological role of interferon-λ3 is currently under intense scrutiny. The mechanism by which IFN-λ3 acts during HCV infection is not yet fully elucidated though this cytokine clearly has direct anti-viral actions in vivo, and readily inhibits HCV replication in hepatoma cells (Huh7.5) (Marcello et al. 2006). A phase II human study of interferon-λ3 for the treatment for HCV is currently underway (Clinical trials identifier: NCT01001754), and whilst it is not yet known if this cytokine administered to patients will be of benefit, it is already clear that future HCV vaccine studies will need to consider the effect of interferon-λ3 host genotype on outcome.

## 1.3 VACCINATION AGAINST HEPATITIS C – BACKGROUND

### 1.3.1 THE IDEAL VACCINE – REQUIREMENTS AND CHALLENGES

Based on the viral-host interactions of HCV infection that have been delineated in the preceding sections, there are 3 characteristic properties that are likely to be shared by successful preventive and therapeutic vaccine approaches alike:

i) These vaccines will need to deal with high levels of viral genetic diversity both between and within hosts- as such, vaccines that target relatively conserved viral regions will be required.

ii) The 'magnitude' of antiviral immunity associated with viral control is not precisely defined. Nevertheless it is likely that a robust, broad and functional T cell and possibly also a humoral immune response will be required

iii) Clearly to be safe, a successful vaccine will need to eradicate HCV from the liver without inducing liver immunopathology. Human studies to date suggest that this is a realistic goal.

**AND THE CHALLENGES...**

In addition to overcoming the diverse mechanisms by which HCV evades immune clearance, there are practical obstacles that have hindered the development of both a preventive and therapeutic Hepatitis C vaccine. HCV is highly fastidious and there is no readily accessible animal model of infection. In 2003, study of viral entry and antibody-mediated neutralisation was enabled for the first time through the development of retroviral particles pseudotyped with HCV envelope glycoproteins (Bartosch, Dubuisson, et al. 2003b). It was not until 2005 that a successful tissue model of HCV infection was developed (Heller et al. 2005). This model uses the human hepatoma cell line Huh7 and a unique viral variant capable of ongoing viral replication. This discovery allowed researchers to characterise the viral life cycle and viral-host interactions during infection

for the first time. Transgenic mice expressing human MHC class I molecules have been used for specific HCV epitope analysis. SCID mouse models with chimeric human livers have been used to evaluate in vivo effects of antibody to envelope glycoproteins (Mercer et al. 2001).

To date the only immunocompetent animal model for the pathogenesis or immune control of viral infection is the chimpanzee. This model has proved very useful in the pre-clinical phases of vaccine development since the immune mechanisms associated with viral control are broadly similar. For example, a study by Folgori was clearly able to demonstrate a reduction in HCV viremia, associated with the induction of robust immunity using a small number of animals (Folgori et al. 2006a). This study subsequently facilitated the use of adenoviral vector technology into human trials. However, there are practical, financial, and for some ethical limitations in using these animals for research (see Bettauer 2010 ). As a result, the small number of chimpanzees that can be used in HCV studies may limit the power of any conclusions that can be drawn.

Designing clinical studies in humans to evaluate a prophylactic vaccine is also challenging since the incidence of HCV infection in developed countries is relatively low other than in intravenous drug users (IVDUs), and men who have sex with men (MSM) - and targeting these patient groups, particularly IVDUs raises various ethical and practical challenges (see Maher et al. 2010 for further discussion). Large studies in developing countries where the incidence may be higher raises logistical difficulties. Assessing the efficacy of a prophylactic HCV vaccine will require large numbers of patients and careful follow up, since acute infection is often asymptomatic. Furthermore, it is possible that a prophylactic vaccine may be unable to achieve sterilising immunity. However, a vaccine that led to an attenuated course of acute infection associated with viral clearance may be sufficient to

ultimately prevent chronic infection, as has been suggested by studies in the chimpanzee model (Folgori et al. 2006a). This also has study design implications; careful consideration must be given to the timing of interferon therapy during primary infection, in the context of a clinical study that is assessing vaccine efficacy. In theory the vaccine may facilitate viral clearance weeks after current guidelines suggest interferon treatment should be given.

### 1.3.2 VACCINE APPROACHES: CURRENT STATUS

Over the last decade numerous HCV vaccine approaches have been assessed in mice and primates. Only a small fraction of animal HCV vaccine studies have progressed to human trials. The majority of these trials have evaluated potential therapeutic vaccines in HCV infected patients. A smaller number have assessed vaccine in healthy volunteers; either with the aim of developing a prophylactic HCV vaccine, or as a bridge to evaluating vaccine in HCV infected patients.

The question as to which HCV antigen a vaccine should target is a key one. The envelope region, that is essential for viral cell entry, may seem the obvious target for a prophylactic HCV antibody inducing vaccine- but as discussed above the major antigenic determinants of the envelope protein are hypervariable both between, and within infected individuals. Chimpanzee data have shown that the induction of HCV envelope antibodies will afford protection with challenge with homologous viral strains only (Farci et al. 1996). This view has been recently challenged using collated chimpanzee data that has demonstrated protection from heterologous viral strains (both genotype 1a) (Houghton & Abrignani 2005; Houghton 2011). Furthermore, cross-genotype neutralising antibodies have been demonstrated using a SCID mouse model transplanted with human hepatocytes (Law et al.

2008). A prophylactic vaccine that induces anti-envelope immunity and attenuates the course of primary infection either alone, or in combination with other approaches remains an attractive goal.

The HCV core protein might seem the obvious candidate for a therapeutic T-cell vaccine, since this is the most highly conserved region of the translated HCV genome both within and between different HCV genotypes. However, studies have shown that the core protein can interfere with innate and adaptive anti-HCV immune responses (Sarobe et al. 2003; Large et al. 1999) (see Figure 1-4, page 27). Furthermore, data suggests that in persistent infection anti-core T cell responses are frequently detected in the absence of viral escape. Although these are typically CD4+ T cell responses, this nevertheless suggests that T cell responses, are unable to control viral replication (Semmo, Barnes, et al. 2005a). Many core based DNA vaccines have been tested in small animal models, and clearly robust anti-core cellular responses can be generated. However, a heterologous prime-boost (plasmid encoding DNA)/ boost (with recombinant protein) vaccination strategy in chimpanzees vaccinated with core, E1, E2 and NS3 failed to induce any anti-core responses (Rollier et al. 2004).

For these reasons, the most recent strategies have focused on inducing T cell responses to the non-structural HCV antigens-which are genetically conserved compared to the HCV envelope, and which are known to contain multiple $CD4^+$ and $CD8^+$ T cell epitopes (Wedemeyer 2009).

Four main vaccine strategies have been investigated in human clinical studies; i) recombinant protein vaccines, ii) peptide vaccines, iii) DNA vaccines, and iv) vector

vaccines. The advantages and limitations of each of these approaches, in combination with a summary of human trials in HCV vaccine development will be outlined below.

### *i) Recombinant protein vaccines*

The use of recombinant proteins as potential vaccine candidates assumes that inducing an immune response to a limited number of viral epitopes is sufficient to develop protective immunity. The principle of this approach is to isolate the gene(s) encoding the appropriate protein and clone it in bacteria, yeast or mammalian cells. Recombinant proteins are prepared either from culture medium or transfected cells. While some recombinant proteins are sufficient in isolation to elicit a strong immune response, others require adjuvant therapy. Generally, protein based approaches induce antibody and $CD4^+$ T cell responses and these have been targeted at either HCV envelope proteins or core proteins.

#### *Envelope protein vaccines*

The Hepatitis B vaccine was the first successful recombinant protein vaccine used in humans. This vaccine employs a Hepatitis B surface antigen that is conserved and is effective in preventing hepatitis B infection through the production of antibodies. In contrast the genetic variability of the HCV viral envelope, that is the main target for anti-HCV antibodies, makes such an approach for a HCV vaccine challenging. Nevertheless, recognition that the presence of pre-existing antibodies to HCV envelope proteins is associated with a better response to PEG-IFN therapy (Depraetere et al. 2000), and that anti-envelope antibodies can lead to an attenuated course of primary infection (Choo et al. 1994) have led to therapeutic and prophylactic vaccine studies respectively, that aim to induce anti-envelope antibodies (Table 1-2, page 48).

*a) Envelope proteins as prophylactic vaccines*

The only published clinical trial of a prophylactic vaccine for HCV utilised a recombinant E1/E2 heterodimer adjuvanted with adjuvant MF59C (an oil in water emulsion) (Frey et al. 2010). This placebo-controlled, dose-escalation phase I study evaluated the vaccine in 60 healthy subjects. All subjects developed neutralising antibodies and T cell lymphocyte proliferation responses to envelope proteins 1 and 2 (E1/E2), and an inverse response to increasing amounts of antigen was noted. The vaccine was well tolerated. Although the study authors suggest that larger clinical trials to evaluate vaccine efficacy are indicated, none have yet been initiated. This is likely because manufacturing E1/E2 in sufficient amounts is a significant obstacle to the widespread application of this approach (personal communication with Dr Antonella Folgori, Okairos).

*b) Envelope proteins as therapeutic vaccines*

The first candidate therapeutic vaccine for hepatitis C was administered to humans in 2003 (Leroux-Roels et al. 2004; Nevens 2003). The vaccine consisted of a recombinant HCV-E1 protein in alum adjuvant. It was administered over 6 months via multiple injections to 20 healthy, and 34 chronically infected, treatment naïve patients. The vaccine induced HCV specific antibody and T cell responses in both patient groups (50/54). Assessment of efficacy showed no change in HCV RNA levels, but in some subjects improvements in liver histology was seen; twenty-four HCV infected patients underwent liver biopsies before and after vaccination. In 9 of these patients, there was histological improvement after 17 months. The observed increase in anti-E1 antibody levels correlated with improvement in liver histological scores and reduction in serum alanine transaminase levels (a measure of liver inflammation).

As a result, this work progressed to a placebo controlled, multicentre trial that evaluated 122 patients who received 4 courses of 6 injections over 3 years. Humoral and cellular immune responses to the E1 protein were induced but vaccination did not prevent histological progression of liver disease. The work was presented in abstract form only in 2008. Innogenetics, the company investigating this vaccine ceased its program in 2008 and no further work has been published.

***Core protein vaccines***

Heat killed yeast cells (*Saccharomyces cervisiae*) expressing conserved core-NS3 fusion protein have been trialed as a therapeutic vaccine candidate (GI5005) (Habersetzer et al. 2009). In a phase-II, placebo controlled trial GI5005 was combined with standard therapy (PEG-IFN/ribavirin) in 66 chronic HCV genotype 1 infected patients. The protocol consisted of a 12-week run-in of standard therapy, followed by weekly doses for 5 weeks followed by monthly doses for 2 months of GI-5005 vaccine, administered subcutaneously. Prior non-responders received 72 weeks, while treatment naïve patients received 48 weeks standard therapy. No data on immunological response has been published. The investigators report an increase in SVR rates in patients homozygous for the interferon-lambda3 risk alleles (oral presentation AASLD 2010). No end of study, peer-reviewed data has been published.

A vaccine using conserved HCV core protein with an adjuvant composed of saponin, cholesterol and phospholipid, called ISCOMATRIX has been evaluated in a phase 1 trial of 30 healthy volunteers (Drane et al. 2009). The vaccine was safe and all 8 volunteers who received the highest dose (50 μg) developed a specific humoral response to the core protein. However, HCV specific $CD8^+$ T cells could only be detected in 2 patients. Further

studies are planned by the same investigators to evaluate this approach as a therapeutic vaccine in HCV infected patients.

Table 1-2; Recombinant protein vaccine studies

| Structure (Investigator) | Phase (year) | Subjects | Outcome | Ref. |
|---|---|---|---|---|
| HCV E1 + alum adjuvant (Innogenetics/ GenImmune) | I (2003) | 20 healthy volunteers + 34 HCV infected, treatment naïve patients | Well tolerated. No HCV viral load change. 50/54 pt T-cell response. Humoral response correlated with improvement in ALT and liver histology | [1] [2] |
| | II (2008) | 122 HCV infected patients randomised (3:1) | Well tolerated. Induced humoral and cellular immune responses. No change in histological progression of liver disease over 3 years | [3] |
| HCV E1/E2 glycoproteins/MF59 adjuvant (Novartis) | I (2010) | 60 healthy volunteers | Well tolerated. Induced antibody and E1/E2 specific T cell responses in all patients independent of vaccine dose. | [4] |
| GI5005: Recombinant yeast transfected with HCV NS3-core fusion protein (Globeimmune) | II (2009) | 66 patients with chronic HCV-G1 (treatment naïve and non-responders) | 17% SVR with vaccine + standard care, compared with standard therapy response of 5% in prior non-responders. | [5] |
| HCV core protein/ISCOMATRIX® (CSL Ltd) | I/IIa (2009) | 30 healthy volunteers | Well tolerated, mild local redness; all developed antibody response. | [6] |
| Advantages: Well tolerated with low toxicity; Induce cross-neutralising antibodies; Proof of concept (HBV vaccine) Limitations: Generally only weak T cell responses elicited. [1] Leroux-Roels et al. 2004 [2]Nevens 2003 [3]Wedemeyer 2008 [4] Frey et al. 2010 [5] Habersetzer et al. 2009 [6] Drane et al. 2009 | | | | |

### *ii) Peptide vaccines*

Like recombinant protein vaccines, peptide based vaccines are well tolerated. They induce HCV specific T cell immunity through the direct presentation of vaccine peptide to the T cell receptor via HLA molecules. However, the major limitation of this approach is that peptide vaccines are HLA specific and as such coverage will be restricted to a subset of the population. Additionally HCV peptide vaccines to date have included a handful of peptides only –and the breadth of the T cell response so induced may be insufficient to control infection. In addition, some peptides may potentially induce tolerance of effector cells or Treg cells rather than inducing immunity (Kaneko et al. 1997). See Table 1-3, page 51 for a summary of the clinical trials outlined below.

IC41 is a peptide vaccine currently in clinical development. It consists of 5 synthetic peptides from core, NS3 and NS4 proteins that are conserved across HCV genotypes 1 and 2, combined with the adjuvant poly-L-arginine. The peptides include 3 $CD4^+$ T cell and 5 HLA A2 restricted $CD8^+$ T cell HCV epitopes. In a phase II, double-blind study, this vaccine was administered subcutaneously to 36 HLA A2 patients with genotype 1 chronic HCV infection who had previously been non-responsive to PEG-IFN/ribavirin and compared with 24 controls (Klade et al. 2008). The vaccine was well tolerated with no serious adverse events. Weak HCV-specific T cell proliferative and IFN-γ ELISpot responses were observed in 67%, and 42% of patients respectively. Three responders with the strongest IFN-γ secreting T cell response had a transient decline in serum RNA (>1 log). In a subsequent, phase II study combining IC41 with PEG-IFN/ribavirin therapy in 35 patients with HCV genotype 1 infection, T cell responses were observed in 73% of vaccinated patients and associated with higher rates of viral clearance (Wedemeyer et al. 2009). The lack of an unvaccinated control arm makes it difficult to draw any significant conclusion regarding vaccine efficacy.

More recently, bi-weekly intradermal IC41 administration was found to induce stronger T cell responses compared with the original monthly subcutaneous injection approach (Firbas et al. 2010). This optimised vaccine schedule has been tested in 50 patients with CHC. A significant decline in viral load was observed after 4 months according to results currently presented in abstract form only (Klade CS 2009). Although Intercell AG, the company developing IC41 announced plans for a phase II trial to begin in 2011, no such study has yet been registered with *clinicaltrials.gov*.

In 2009, a peptide derived from HCV core region (C35-44) was evaluated in a phase I dose-escalation Japanese study of 26 patients (23 non-responders to PEG-IFN/ribavirin and 3 who had declined standard therapy) (Yutani et al. 2009). A series of 6 biweekly subcutaneous injections was sufficient to induce peripheral peptide specific $CD8^+$ activity in 15/25 patients (measured with ELISpot) and 12 injections augmented peptide specific IgG production. A >30% improvement in ALT was observed in 7/24 patients and 2 patients had a >1 log reduction in viral load. Further evaluation with a phase II study is under consideration.

Another Japanese study adopted a 'personalised' peptide vaccine approach. In this study 12 patients with HCV genotype 1b who had previously failed PEG-IFN/ribavirin therapy, were administered 4 $CD8^+$ A24 peptides in combination with Freund's adjuvant. Only those peptides that induced an immune response in each individual following the first vaccine dose were then administered fortnightly for another 14 vaccinations (Yutani et al. 2007). At the first assessment (following 7 vaccine doses), the majority of patients had developed peptide specific T cell responses. A dose dependent decrease in serum ALT and HCV RNA levels was observed in 5 and 3 patients respectively.

A novel method of peptide delivery using autologous monocyte derived dendritic cells was recently explored in an Australian study (Gowans et al. 2010). Dendritic cells were first harvested and then loaded and activated ex vivo with HLA A2 1-restricted T cell epitopes. 6 patients chronically infected with HCV who had previously failed PEG-IFN/ribavirin therapy received vaccine. All patients developed weak de novo HCV-specific $CD8^+$ T cell responses (measured by IFN-γ ELISpot assays). The authors hypothesise that T cell responses induced to viral epitopes not included in the vaccine may have been caused by

epitope spreading. There was no change in viral load or anti-HCV core antibody levels and T cell responses were not sustained.

Finally, a phase I, placebo controlled trial assessing a virosome-based vaccine containing NS3 peptides was completed in 2010 but no data released. (ClinicalTrials.gov Identifier: NCT00445419)

In summary, peptide based vaccines are well tolerated and able to induce weak peptide specific T cell and humoral responses. Efficacy needs to be optimised, as trials show a significant reduction in viral load in only a few patients.

Table 1-3; Peptide HCV vaccine studies

| Structure (Investigator) | Phase (year) | Subjects | Outcome | Ref |
|---|---|---|---|---|
| IC41: 5 peptides from core, NS3 and NS4 + poly-L-arginine adjuvant, (Intercell AG) | II (2008) | 60 chronic HLA-A2 HCV infected non-responders | Well tolerated. 67% T cell response. Transient serum HCV RNA decline (>1 log) in 3 pts | [1] |
| Peptide derived from core protein (C35-44) (Karume University) | I (2009) | 26 patients with chronic HCV (23 non-responders, 3 untreated) | Well tolerated.15/25 responded, 2/25 1 log decline in HCV RNA | [2] |
| Personalised peptide approach $CD8^+$ A24 peptides + Freund's adjuvant (Karume University) | I (2007) | 12 chronic HCV-1b infected non-responders | Well tolerated. Majority developed peptide specific T cell responses. 5pts reduced ALT and 3 pts had a HCV RNA decline | [3] |
| Autologous dendritic cell delivered HLA A2 epitopes (Burnet Institute + others) | I (2010) | 6 chronic HCV infected non-responders | Well tolerated. Transient T cell responses | [4] |
| NS3/Virosome, (Pevion Biotech) | I (2010) | 30 healthy Volunteers | No results published | n/a |

Advantages: well tolerated with low toxicity; induces both cellular and humoral responses
Limitations: Limited immunogenicity (few epitopes only); need to tailor to patient HLA type; concern regarding appropriate peptide choice (eg. induce Tregs rather than $CD8^+$ cells). n/a= Not available
[1] Klade et al. 2008 [2] Yutani et al. 2009 [3] (Yutani et al. 2007) [4] Gowans et al. 2010

### *iii) DNA vaccines*

In 2001, the first DNA vaccine was licensed for use to protect horses from the West Nile virus (B. S. Davis et al. 2001). Initial work in the decade leading up to this significant accomplishment, demonstrated that injection of a plasmid containing a gene could effectively result in protein expression in vivo and subsequently induce a host immune response (Boyer et al. 2007; Boyer et al. 2005). Substantial research efforts have been aimed at developing an effective Hepatitis C DNA vaccine. Related clinical trials are summarised in Table 1-4, page 54).

Unfortunately, the initial success observed with DNA vaccination induced immunisation in mice did not translate well into similar results in humans – likely, in part, because in larger hosts, the efficacy of vaccine DNA uptake and gene expression is reduced (B. Wang et al. 1993; H. L. Davis et al. 1993). Subsequently several methods were developed to improve DNA delivery and hence immunogenicity. These methods include:

(i) Biolistic technology (biological ballistic or 'gene gun') – Tungsten particles are loaded with genetic material and using a device known as a particle gun fired at living plant cells (Yang et al. 1990). This resulted in delivery of DNA into a proportion of plant cells. In 2000, a clinical trial using a gene gun for DNA vaccination against Hepatitis B was performed with successful induction of protective humoral immunity as well as Hepatitis B surface antigen specific T cell responses (Roy et al. 2000).

(ii) Electroporation (EP)– Electrical impulses create transient pores in living cells and subsequently allow delivery of DNA across the cell membrane. The local damage to cell membranes is also thought to enhance the local inflammatory response

(Mathiesen 1999; Gronevik et al. 2005; Peng et al. 2007). EP, at least in mice is said to enhance the immunogenic response by DNA vaccination 10-fold.

The first DNA based vaccine to reach clinical trial for HCV infection did not employ either of these adjuvant vaccine delivery techniques. This phase 1 trial based in Cuba evaluated a vaccine (CICGB-230) combining plasmid expressing HCV structural antigens (core/E1/E2) with recombinant core protein (Co.120) (Lajonchere & Shoukry 2009). 15 patients with HCV genotype-1 infection who had previously failed PEG-IFN/ribavirin therapy received monthly intramuscular injections for 6 months. The vaccine was well tolerated. The T cell response to the vaccine components (as well as NS3) was measured using IFN-γ ELISpot and proliferation assays one month following the final vaccine. Although low levels of T cell immunity were observed in 11 patients, others showed a reduction in responses. Six patients developed weak de novo neutralising antibody responses against heterologous viral pseudoparticles. Only one patient had a drop in viral load of greater than one $\log_{10}$. In addition, the authors reported stabilisation or improvement in liver histology –however, the absence of a control arm, makes this finding difficult to interpret.

The second HCV DNA based vaccine (ChronVac-C) to reach human trials employed electroporation to enhance the immunogenicity of intramuscular injection of plasmid expressing HCV antigens NS3/4a. Extensive codon modification was undertaken to allow effective DNA expression and enhance in vivo T cell response. Twelve, treatment naïve, genotype-1 HCV infected patients with a low viral load (< 800,000 IU/ml) received 4 monthly doses of DNA (3 groups: 167 μg, 500 μg and 1500 μg) in this phase I/IIa clinical trial. Preliminary results from this trial were reported in 2009 (Sallberg 2009). 67% (4/6) of patients who received the higher doses had reductions in viral load exceeding 0.5 $\log_{10}$

lasting for 2 to more than 10 weeks, with corresponding activation of T cell responses in 3 of these patients. No severe adverse reactions were observed.

Table 1-4; DNA vaccine studies*

| Structure/Vector (Investigator) | Phase (year) | Subjects | Outcome | Ref |
|---|---|---|---|---|
| CICGB-230: Plasmid core/E1/E2 + recombinant core protein. (University of Montreal + others) | I (2009) | 15 genotype 1 infected non-responders | Well tolerated, No viral clearance, 11/15 - Weak specific T-cell responses (ELISpot and proliferation). 6/15 - Weak humoral response | [1] |
| ChonVac-C: Plasmid NS3/4a + electroporation (Tripep) | I/IIa (2009) | 12 HCV genotype 1 infected, treatment naïve patients | Well tolerated. 4/6 who received higher doses: Viral load reduction >0.5 log with corresponding T-cell response in 3 patients | [2] |

*Ideal genes should represent part of the genome with limited genetic variability and maximal immunogenicity
Advantages: present a broad range of epitopes. ELISpot: Enzyme-linked immunosorbent spot
Limitations: Electroporation is painful
[1] Lajonchere & Shoukry 2009 [2] Sallberg 2009

### *iv) Vector based vaccines*

The use of viral vectors for the delivery of DNA corresponding to viral sequences is an appealing vaccine choice. Adenoviral vectors have shown to be potent inducers of HCV specific T cell responses in the chimpanzee model and to reduce peak HCV viremia during primary infection (Folgori, Capone, et al. 2006a). This approach may induce a broader range of viral epitopes than a peptide based approach since the immunogen contained within the vaccine is not HLA restricted.

Modified Virus of Ankara (MVA) is a highly attenuated poxvirus strain that has been used safely in several vaccine designs for conditions such as HIV, colorectal cancer, tuberculosis and melanoma. A therapeutic vaccine (TG4040) using MVA that expresses NS3/4/5B proteins has been evaluated for safety and immunogenicity in an open-label, multicenter, dose-escalation study (Habersetzer et al. 2011). 15 chronically infected HCV patients received 3 weekly injections, 9 of whom received a 4$^{th}$ injection at 6 months. 5/15 had cellular immune responses detectable following vaccination (2 of these patients had pre-

existing but responses of lower magnitude prior to vaccination). 8/15 patients showed a transient decline in HCV viral load (0.5 - 1.4 $\log_{10}$). The authors suggest there is a temporal relationship between the viral load nadir at day 37 and peak cellular immune responses, but no statistical correlation is presented. Furthermore, no autologous viral sequence data is presented to provide evidence that vaccine induced T cell responses actually target circulating virus. It is possible that the observed change in viral load is explained by natural longitudinal variations in viral load that have been reported in chronic HCV infection as > 1 $\log_{10}$ in some instances (Gordon et al. 1998).

A phase II trial using the TG4040 vaccine in ongoing - interim results were presented at the EASL conference in 2012 (ClinicalTrials.gov Identifier: NCT01055821). 154 treatment naïve patients were divided into 3 treatment arms: 1 control arm (Arm A) treated with PEG-IFN/ribavirin alone, and 2 arms (B and C) that received 2 different vaccination schedules together with PEG-IFN/ribavirin – Arm C patients were vaccinated 6 times before beginning therapy whilst Arm B patients received only one vaccination prior to commencing PEG-IFN/ribavirin therapy. Interim data indicate vaccination is well tolerated and that higher EVR rates (virus negative after 12 weeks PEG-IFN/ribavirin therapy) in vaccinated patients (Arm C only) than controls. However, after 24 weeks therapy, there was no significant difference between the control group and vaccinees as defined by viral clearance. End of treatment response rates and SVR data are not yet available. No data evaluating cellular immune responses to vaccination was presented.

Data from our own group and others (Adrian Hill, Oxford) indicate the use of MVA vaccination alone is insufficient to induce detectable T cell responses against vaccine immunogen (unpublished data) because MVA is ineffective at priming T cells. This raises

questions about the mechanism behind the apparent success of TG4040 - final trial data investigating immune responses to vaccination is awaited.

The Oxford vaccine group is investigating adenovirus and MVA vectors as vaccines for HCV – this will be discussed in further detail in the following section (see Section 1.4).

Table 1-5; Viral vectored HCV vaccine studies

| Structure/Vector (Investigator) | Phase (yr) | Subjects | Outcome | Ref |
|---|---|---|---|---|
| TG4040: MVA vector expressing NS3/4/5B proteins (Transgene) | I (2009) | 15 chronically infected HCV patients | 8/15 declined HCV viral load (0.5-1.4 log) and authors suggest there is a temporal relationship with peak cellular immune responses (IFN-γ ELISpot) | [1] |
| | II (2011) | 154 chronically infected HCV patients | Well tolerated. Higher EVR rates in vaccinated group compared with control. No end of treatment results or immunogenicity data yet available | [2] |
| Adenovirus vector (Ad6 and AdCh3) expressing NS3-5B proteins (Okairos and Oxford university) | I (2009) | 36 healthy volunteers | Highly immunogenic and well tolerated | [3] |

Advantages: Efficient induction of cellular immune responses; presentation of a broader range of viral epitopes. Limitations: Pre-existing immunity to viral vector (adenovirus)-may be overcome by the use of rare serotypes; limited experience in humans; potential for dissemination in immunodeficient patients (vaccinia virus). cEVR = complete early virological response = virus negative after 12 weeks therapy by PCR.
[1] Habersetzer et al. 2011 [2] Wedemeyer et al. 2012 [3] Barnes et al. 2012

## 1.4 SUMMARY OF VACCINE APPROACHES AND OXFORD HCV VACCINE PROGRAM

Table 1-6 presents a summary of vaccine approaches under investigation.

| Table 1-6 Summary of vaccine approaches | |
|---|---|
| Vaccine strategy | Major limitations/promise |
| Recombinant protein based vaccines | - Failure to stimulate $CD8^+$ T cell responses<br>- Might provide a useful adjunct to a T cell vaccine (such as a viral vector vaccine) |
| Peptide based vaccines | - Poorly immunogenic<br>- Applicability is limited by the HLA repertoire of the vaccinated host |
| DNA based vaccines | - In isolation may not be sufficiently immunogenic (Electroporation or other adjuncts may overcome this barrier)<br>- Combination with viral vector vaccines is promising |
| Viral vector based vaccines | - MVA – in isolation, multiple injections required and T cell priming is poor<br>- Ad/MVA heterologous approach provides promise as it overcomes issues with poor MVA T cell priming |
| Adjuvants provide a potential means of improving the immunogenicity of these approaches | |

A vaccine that induces strong, and broad T cell responses to HCV is an ideal goal, and a virally vectored approach is one of the most promising methods for achieving this. The Oxford HCV vaccine program is therefore exploring a combination of heterologous viral vectors carrying HCV immunogen for both prophylaxis against HCV infection and therapy. This work is a collaborative effort between Oxford researchers (Barnes/Klenerman group) and an Italy based pharmaceutical company, Okairos. To date, 3 trials have been initiated in Oxford. In brief, these are:

i) **HCV001** – This phase I/II dose escalation study investigated the application of 2 heterologous adenovirus vectors carrying HCV immunogen in 36 healthy volunteers for HCV prophylaxis. Vaccine was well tolerated and immunogenic (Barnes et al. 2012).

ii) **HCV002** – This therapeutic vaccine trial employs the same 2 adenovirus vector vaccines as HCV001 but in patients with chronic HCV infection. Results from this trial are in preparation for publication.

iii) **HCV003** – This trial employs the combination of an adenovirus and an MVA vector as both preventative and therapeutic vaccines. The therapeutic arm of this trial evaluating vaccination in patients with chronic HCV infection is the focus of Chapter 3 of this thesis. It is introduced in detail in the following sections.

## 1.5 THERAPEUTIC HCV VACCINE TRIAL (HCV003)

When designing the HCV003 study, the key questions raised were:

1. What vaccine immunogen should be used?
2. Which viral vectors should be used to deliver the immunogen?
3. To whom should the vaccine be delivered and according to what schedule?
4. What are the potential risks, both of vaccination, and trial participation in general?
5. What should the key objectives be?

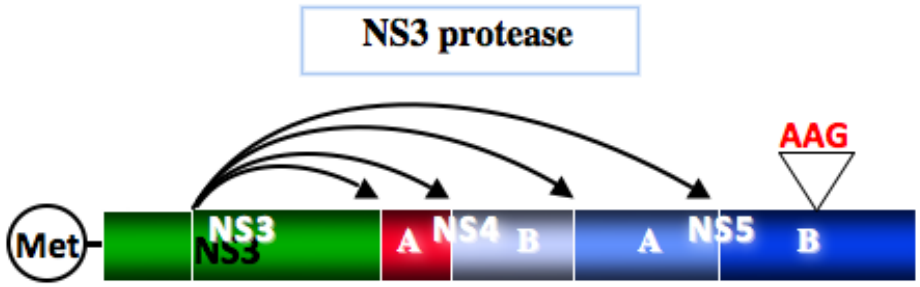
### 1.5.1 WHAT VACINE IMMUNOGEN SHOULD BE USED?

Once the decision to explore viral vectors for HCV vaccination was made, a key question that followed was what insert to choose in order to have the best chance of inducing a protective immune response. The HCV database group has mapped a large number of T cell epitopes in the NS region of HCV (more than 90 T helper and more than 70 CTL (cytotoxic T lymphocyte epitopes) (Kuiken et al. 2005). In addition, published data in chronically and acutely infected patients suggest a predominant role for epitopes mapped in the NS region in inducing T cell immunity (Bowen & C. Walker 2005; Lauer et al. 2002;

Spada et al. 2004). For these reasons, an insert was chosen comprising NS amino acid sequences (Figure 1-6). This sequence was taken from the genotype 1, subtype b, BK strain isolate.

To improve vaccine safety a mutation inactivating the enzymatic activity of the encoded HCV polymerase gene was introduced, eliminating any potential replication capability of the vaccine - The original amino acid sequence GlyAspAsp at positions 1711 to 1713 in the catalytic site of the NS5b insert was replaced with the inactive AlaAlaGly sequence (Capone, Meola, et al. 2006a). This genetic inactivation of NS5b does not reduce gene expression or polyprotein processing by the NS3 protease present in the encoded HCV fragment (Capone, Zampaglione, et al. 2006b).

Figure 1-6; The non-structural (NS) regions of HCV encoded by the vaccine.




Different colours represent different NS regions. The vaccine insert spans the NS3-NS5B regions of the HCV virus taken from genotype 1b BK strain. AAG = AlaAlaGly sequence, a mutation introduced in the NS5b region to inactivate the viral polymerase.

## 1.5.2 WHICH VIRAL VECTOR SHOULD BE USED TO DELIVER VACCINE IMMUNOGEN?

### *Adenovirus vectors*

The decision to use an adenovirus as one vector for delivery of vaccine immunogen in the HCV003 trial was based on a significant body of preclinical and clinical data that has conclusively shown adenovirus-vectored genetic vaccines to be superior to most alternative vectors.

From a safety point of view, despite adenoviruses infecting large numbers of cells, the transferred genetic information remains epichromosomal thus avoiding the risk of insertional mutagenesis. Several clinical trials investigating adenoviral vectors for transgene expression as a potential therapy for cystic fibrosis demonstrated that insert expression is short-lived (undetectable at 40 days) and potently immunogenic – both obvious problems for cystic fibrosis therapy- but not necessarily so for vaccination, where potent immunogenicity is ideal, and long-lived expression of insert may not be required once a memory T cell population is established (T. W. R. Lee et al. 2005; Robbins & Ghivizzani 1998).

Extensive phase I and more recently, phase II clinical trials using replication-defective Ad5 vectors have been undertaken in HIV-1 vaccine research with excellent safety profiles (Duerr et al. 2006; Cohen 2005; Robertson 2005; Shiver & Emini 2004). In addition, previous mass vaccination campaigns in USA military personnel, using orally administered live human adenovirus serotypes 4 and 7 have shown good safety and efficacy data.

Preliminary work by our collaborators in Italy (Okairos), constructed Ad5-based replication defective vectors encoding different HCV antigens and showed they are very

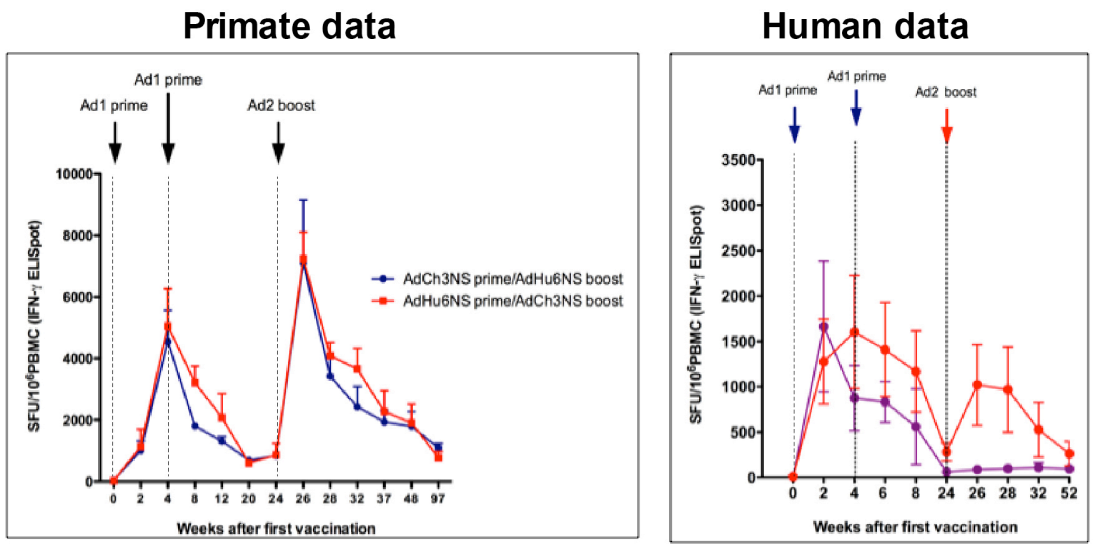
potent vectors for the induction of protective HCV-specific T cell responses in non-human primates (Capone, Meola, et al. 2006a).

Because adenovirus is a ubiquitous infection in man, it is not surprising that a major limiting factor to the usage of human adenovirus vectors is the levels of anti-vector immunity already present. For example, estimates suggest that, depending on the region, between 45 – 80% of adults carry Ad5 neutralising antibodies (Tatsis & Ertl 2004). To overcome the problem of pre-existing anti-adeno vector immunity, in our earlier trials we chose adenoviruses derived from chimpanzees (ChAd3) and rare human serotypes (Ad6) to which humans are only rarely exposed.

As previously described, the Barnes/Klenerman group recently published a Phase I trial (HCV001) using a combination of ChAd3 and Ad6 vectors encoding the NS antigen (NSmut) in healthy volunteers, and work is ongoing using these vectors in HCV infected patients (study HCV002TV) (Barnes et al. 2012). These 2 studies have employed the identical ChAd3-NSmut vaccine that will be used in the HCV003 study, and demonstrate that it is both safe and immunogenic.

Work in macaques demonstrated prime/boost vaccination using heterologous adenovirus vectors generates an effective boost vaccine response (Figure 1-7). However, an unexpected and significant problem in the HCV001 study was blunting of the HCV-specific immune response after boost vaccination.

Figure 1-7; Magnitude and kinetics of T cell responses after vaccination with prime/ boost vaccination in primates (left graph) and healthy volunteers (right graph).




The peak response in humans following boost vaccination is blunted compared with primate data. The y-axis is mean (±SEM) summed ex vivo IFN-γ ELISpot responses to NS peptides in the vaccine immunogen. Primate data based on 3-4 macaques/group. Human data based on 5 volunteers/group (from HCV001 trial). Red lines indicate groups who receiving Ad6 for prime vaccination followed by AdCh3 boosting. The blue lines represent groups who received the vectors in the reverse order. Vertical dotted lines indicate timing of prime/boost vaccination relative to study commencement.

Further evaluation demonstrated that cross-reactive anti-vector antibodies induced at priming were likely to be limiting boosting efficacy. To overcome this problem, the HCV003 trial was designed to employ a second unrelated viral vector in place of the AdHu6-NSmut vaccine used in the earlier trials. ChAd3 rather than Ad6 was selected as the priming vector for HCV003 because pre-existing antibodies to Ad6 are found in approximately 22% whereas ChAd3 has a seroprevalence of only 12% (Colloca et al. 2012).

### *Modified Vaccinia Ankara vector*

The successful worldwide eradication of smallpox via vaccination with vaccinia virus highlighted vaccinia as a possible candidate vector for vaccine developers. Despite the safe use of replication-competent vaccinia virus for small pox eradication, the development of

attenuated vaccinia strains further improved potential vaccine safety. Modified vaccine Ankara (MVA) is one such strain, originally derived by more than 500 serial passages in primary chicken embryo fibroblasts.

MVA has been safely used in humans - from 1972 until 1980 (the end of compulsory smallpox vaccination) MVA was part of Germany's official immunisation schedule (Mahnel & Mayr 1994). Over 120,000 individuals were vaccinated and closely followed in a large field study of this vaccine – typically a small red nodule (up to 4 mm) developed at the site of vaccination on day 4 or 5 and only a small number of vaccinees developed systemic side effects such as fever over 38.5°C (Mayr et al. 1978). In this study MVA also proved to be non-contagious and non-virulent.

For the reasons given above, MVA was considered to be an attractive alternative vaccine vector to investigate in the HCV003 trial. Theoretically, the use of this new vector should overcome the problem of blunting of boost responses encountered due to cross-reactive anti-vector immunity in the HCV001 and HCV002 trials.

The MVA-NSmut candidate vaccine to be trialed in the HCV003 study has not previously been administered to humans. Nevertheless, at the time of study initiation, the safety profile for the vaccine could be partially deduced from a number of clinical trials using recombinant MVA vectors expressing different antigens (eg. ME-TRAP study codes VAC033 and Mal034) that had already taken place in Oxford. To date there have been no vaccine-related serious adverse events in any of these trials (personal communication with Prof. Adrian Hill, director of Jenner Vaccine Institute, Oxford).

### 1.5.3 TO WHOM SHOULD THE VACCINE BE ADMINISTERED AND ACCORDING TO WHAT SCHEDULE?

For the HCV003 trial, it was decided to combine experience from the earlier 2 Oxford based HCV vaccine trials (HCV001 and HCV002) and evaluate ChAd3-NSmut/MVA-NSmut vaccination both in healthy volunteers (Arm A), and in patients with chronic HCV infection (Arms B and C). This permitted the evaluation of vaccine immunogenicity in both the prophylactic and therapeutic setting, and direct comparisons to be made between vaccine responses in healthy individuals and those with chronic HCV infection.

Details of study arms, numbers of individuals to be recruited, doses and timing of vaccination are described in Methods Section 2.1.1 (page 70). There is however, one important point that should be highlighted here relating to study protocol. There is evidence, at least in mice, that reducing viremia during chronic infection may improve T cell responses to vaccination by reducing constant antigenic stimulation, and therefore T cell exhaustion (Wherry & Ahmed 2005) – this is the reason behind dividing HCV infected trial participants into several groups (B1, B2 and C). Arm B patients received vaccination either early (B2), or late (B1) during PEG-IFN/ribavirin therapy, so that the effect of reducing viremia on vaccine response could be evaluated. Meanwhile, for comparison Arm C patients received vaccination alone without concurrent PEG-IFN/ribavirin therapy.

## 1.5.4 WHAT ARE THE POTENTIAL RISKS OF VACCINATION/TRIAL PARTICIPATION?

### *Local reactions to vaccine/venipuncture*

No serious local AEs have previously been documented using the IM route in our earlier HCV001 or HCV002 trials. Based on these studies it was considered likely that vaccination would precipitate a local inflammatory reaction characterised by mild erythema, tenderness and swelling.

### *Systemic reactions to vaccine*

As with any vaccine, systemic reactions including a flu-like illness with low-grade fever, chills and malaise were considered possible AEs. More serious adverse effects such as Guillain-Barre syndrome or serious allergy were considered possible but unlikely. Safety data from our recently published HCV001 trial (using the ChAd3-NSmut vaccine) demonstrated no serious AEs in healthy volunteers. VAC033, an Oxford based study for malaria which used an MVA based vaccine can be used to predict systemic side effects for our MVA-NSmut vaccine – no moderate or severe systemic SEs occurred in patients who received intramuscular MVA vaccination in this trial.

There is a theoretical risk that HCV specific T cells generated by vaccination could target infected hepatocytes and consequently induce significant immunopathology through hepatocyte death and liver inflammation. However, early human trials in HCV infected patients using adenovirus vectors for vaccination in which HCV specific T cells have been successfully induced, have not caused significant liver inflammation measurable by a rise in ALT (HCV002 – unpublished data). Furthermore, severe liver disease in natural acute HCV infection (when robust T cell immunity is induced) is uncommon and fulminant liver failure is exceedingly rare. This is because in HCV infection, unlike hepatitis B infection, only a minority (10%) of hepatocytes are infected (Liang et al. 2009).

### 1.5.5 WHAT SHOULD THE PRIMARY OBJECTIVES OF THE THERAPEUTIC ARMS OF THE HCV003 TRIAL BE?

The primary HCV003 study endpoints were safety and immunogenicity.

Key questions to address were, in HCV infected patients, can a heterologous prime/boost vaccination schedule using ChAd3-NSmut and MVA-NSmut:

1. Safely induce HCV specific T cell responses during PEG-IFN/ribavirin therapy for HCV genotype-1 infection, after a significant decline (> 2 logs) in viral load, 14 weeks into therapy (Arm B1)?
2. Safely induce HCV specific T cell responses during combination therapy for HCV genotype-1 infection, 2 weeks into therapy (Arm B2)?
3. Safely induce HCV specific T cell responses in patients with chronic HCV (not receiving PEG-IFN/ribavirin therapy) and a high viral load (Arm C)?

### 1.5.6 ETHICAL APPROVAL

Approval for the HCV003 study was granted by the Oxfordshire Research Ethics Committee (reference number 10/H0604/45). I obtained written, informed consent from all trial participants.

## 1.6 THE HOST IMMUNE RESPONSE TO STANDARD THERAPY TRIAL (THIRST TRIAL)

The second investigative chapter of this thesis (Chapter 4) is briefly introduced here. Clearly the application of ChAd3-NSmut/MVA-NSmut as a therapeutic strategy for chronic HCV infection may be impacted significantly by changes in immune function driven by both chronic infection itself, and also by PEG-IFN/ribavirin therapy (if vaccination is given concurrently). Indeed, the capacity of such a vaccine to induce an appropriate immunogenic response may be enhanced, or indeed compromised by concomitant PEG-IFN/ribavirin therapy. With this in mind, I designed the THIRST study to evaluate 2 broad questions:

1. Are there any pre-treatment functional, phenotypic and differentiation status changes in immune cell subpopulations during chronic HCV infection?
2. What is the impact of PEG-IFN/ribavirin therapy longitudinally on the function, phenotype and differentiation of immune cell subpopulations?

### 1.6.1 RATIONALE FOR STUDY

Numerous studies indicate that both innate and adaptive immunity is impaired in patients with chronic hepatitis C infection (see Section 1.2, page 33 and Figure 1-5, page 35). Whether this impairment is a cause or a consequence of chronic infection is unknown. Furthermore, the impact of these changes on the outcome of HCV therapy is unknown. Evidence to support a role for host immunity in viral clearance during PEG-IFN/ribavirin therapy is scarce – indirect evidence comes from the fact that patients with impaired immunity such as those with HIV or those on post-transplant immunosuppressive therapy are less successful in achieving an SVR with standard therapy (Chung et al. 2004).

However, these patient groups have high pre-treatment viral loads – an independent factor linked to poor rates of viral clearance (Ishibashi et al. 1995; Shiratori et al. 1997).

Recent work by our group demonstrates that during Hepatitis C treatment with PEG-IFN/ribavirin, a significant proportion of patients develop lymphopenia and this correlates positively with treatment outcome (Humphreys et al. 2012). It may be that lymphopenia is a surrogate marker of appropriate PEG-IFN/ribavirin dosing that is driven by bone marrow suppression. An alternate explanation however, is that treatment-mediated recruitment of circulating lymphocytes to infected hepatocytes assists directly in viral clearance.

So what is already known about the immunomodulatory effects of PEG-IFN/ribavirin and are these likely to impact on treatment outcome or the response to a vaccine delivered during standard treatment? The complex actions of these 2 agents are summarised in detail in Section 1.1.3 (page 22). In-vivo, a number of studies have evaluated the effects of PEG-IFN/ribavirin therapy on specific subsets of immune cells by comparing pre, on and post-treatment activity. However, very few studies have evaluated changes that occur early, during the first four weeks of therapy. The paucity of data during this period is striking because it is clear that early changes in viral kinetics are critical in determining the ultimate outcome of treatment for CHC (Fried et al. 2002; Jessner et al. 2001) – Fried and colleagues demonstrated a viral load decline of < 70% of baseline 24 hours after commencing PEG-IFN predicts non-response with a sensitivity of 83% and specificity of 100%.

With this background in mind, in the second investigative chapter of this thesis (Chapter 4), I chose to evaluate baseline differences in immune cell subsets in HCV infected patients

together with changes that occur early PEG-IFN/ribavirin therapy, to better understand the potential impact these may have on the concurrent administration of a therapeutic vaccine during standard HCV therapy.

### 1.6.2 ETHICAL APPROVAL FOR THE THIRST STUDY

The Oxfordshire Research Ethics committee granted full ethical approval for the THIRST study (study number: 04.0XA.10). Written, informed consent was obtained for all trial participants.

## 1.7 SUMMARY OF INTRODUCTION

In summary, HCV infection represents a significant worldwide problem for which current therapies are suboptimal. Prophylactic and therapeutic vaccines are urgently needed. In this thesis, use of a potent, virally vectored vaccine in humans will be explored. I will assess the immunogenicity and safety of this approach in HCV infected patients for the first time. Since vaccination is administered concurrently with PEG-IFN/ribavirin therapy in some patients, additional work will focus on the effect of these drugs on T cell immunity.

# Chapter 2 Materials and Methods

## 2.1 ESTABLISHING COHORTS OF PATIENTS AND CONTROLS

### 2.1.1 HCV003 TRIAL

Any patient with chronic hepatitis C (CHC) genotype 1 infection who attended the Hepatology outpatient clinic at the John Radcliffe Hospital in Oxford (or High Wycombe Hospital) was considered for enrolment in the HCV003 trial. Following preliminary review in outpatients, any patient who expressed interest in participating in the trial was first provided with a trial information sheet. They were then contacted by phone 1 week later by a research nurse. If at that time they remained interested in participating in the trial, a screening appointment was arranged. Only patients who met the inclusion/exclusion criteria (Table 2-1 and Table 2-2) and gave appropriate consent (Appendix 1) were enrolled.

| Table 2-1; Inclusion criteria for HCV003 trial |
|---|
| HCV genotype-1 infection |
| Patients must not be currently receiving any treatment for HCV infection. |
| Aged 18 to 65 years (inclusive) |
| Resident in or near the trial sites for the duration of the vaccination study |
| For men in Arm B, including those with pregnant partners, a willingness to use barrier contraception until 6 months after completing treatment with PEG-IFN/ribavirin |
| For women in Arm B, of child bearing potential, a willingness to practice continuous effective contraception during the study and a negative pregnancy test on the day(s) of vaccination. |
| For men in Arm C, including those with pregnant partners, a willingness to use barrier contraception until 3 months after the last vaccination |
| For women in Arm C of child bearing potential, willingness to practice continuous effective contraception during the study and a negative pregnancy test on the day(s) of vaccination |
| Patients with HCV in groups B1 and B2 must be treatment naïve to previous PEG-IFN and ribavirin combination therapy. They may be included if they have been previously treated with interferon monotherapy but relapsed after treatment. |
| Patients with HCV in groups C1 may be treatment naïve, or have been previously treated with interferon monotherapy or interferon and ribavirin therapy and relapsed after treatment |

| Table 2-2; Exclusion criteria for HCV003 trial |
|---|
| Participation in another research study involving an investigational product in the 30 days preceding enrolment, or planned use during the study period |
| Prior receipt of a recombinant simian or human adenoviral vaccine |
| Clinical, biochemical (abnormal liver synthetic dysfunction defined by an elevated blood prothrombin time or a low blood albumin level), ultrasonographic, or liver biopsy (histology) evidence of cirrhosis or portal hypertension |
| Any confirmed or suspected immunosuppressive or immunodeficient state, including HIV infection; asplenia; recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (inhaled and topical steroids are allowed) |
| History of allergic disease or reactions likely to be exacerbated by any component of the vaccine, |
| History of clinically significant contact dermatitis |
| Any history of anaphylaxis in reaction to vaccination |
| Pregnancy, lactation or willingness/intention to become pregnant during the study |
| History of cancer |
| Suspected or known current alcohol abuse as defined by an alcohol intake >42 units every week |
| Current suspected or known injecting drug abuse |
| Seropositive for hepatitis B surface antigen (HBsAg) |
| Seropositive for HIV (antibodies to HIV) |
| Any other significant disease, disorder or finding, which, in the opinion of the Investigator, may either put the patient at risk because of participation in the study, or may influence the result of the study, or the patient's ability to participate in the study |
| Any other finding which in the opinion of the investigators would significantly increase the risk of having an adverse outcome from participating in the protocol |
| In addition to the above listed exclusion criteria: |
| Patients with HCV may not enter arms B1 and B2 of the study if any of the following exclusion criteria applies: |
| Patients are non-responders to previous IFN-α monotherapy |
| Patients who received IFN-α and ribavirin or PEG-IFN and ribavirin in the past and who were non-responders or who relapsed during or after therapy |
| Patients with a known allergy to ribavirin or interferon-α |
| Haemoglobin less than 10 g/dl |
| Severe neutropenia or thrombocytopenia |
| Patients who have had a heart attack or who have suffered from any other severe heart disease in the last 6 months |
| Patients with haemoglobinopathies |
| Autoimmune hepatitis or other autoimmune disease |
| History of organ transplantation |
| Uncontrolled seizures |
| Uncontrolled severe psychiatric conditions |
| Patients with HCV may not enter arm C if they were previous non-responders to interferon monotherapy, or interferon and ribavirin combination therapy |

The trial was designed with 3 arms; Arm A were healthy volunteers, Arm B were patients with CHC who received vaccine together with standard PEG-IFN/ribavirin therapy, and Arm C were patients with CHC who received vaccine alone (Table 2-3).

Participants in Arm A (healthy volunteers at low risk of HCV infection) were not recruited or managed by myself and the primary objective of this arm was to evaluate the vaccine as a potential prophylactic vaccine – for these reasons, I have not included details of their recruitment and management in the body of this thesis. Where relevant however, I include data from this group as a means of comparing vaccine responses between healthy individuals and those with chronic HCV infection.

Table 2-3; HCV003 Trial arms

**Arm A: Vaccination in healthy volunteers**

**Group A1:** 4 volunteers; 1 dose MVA-NSmut 2 X $10^8$ pfu

**Group A2:** 10 volunteers; 1 dose ChAd3-NSmut 2.5 x $10^{10}$ vp at week 0 and 1 dose MVA-NSmut 2 x $10^8$ pfu at week 8

**Arm B: HCV infected patients receiving pegylated interferon and ribavirin**

**Group B1:** 4 patients; 1 dose ChAd3-NSmut 2.5 x $10^{10}$ vp at week 14 and 1 dose MVA-NSmut 2 x $10^8$pfu at week 22, after starting PEG-IFN and ribavirin therapy

**Group B2:** 5 patients; 1 dose ChAd3-NSmut 2.5 x $10^{10}$vp at week 2 and 1 dose MVA-NSmut 2 x $10^8$ pfu at week 10, after starting PEG-IFN and ribavirin therapy

**Arm C: Vaccination during chronic HCV infection**

**Group C1:** 4 patients; 1 dose ChAd3-NSmut 2.5 x $10^{10}$ vp 2 weeks after trial enrolment (and collection of baseline bloods) and 1 dose MVA-NSmut 2 x $10^8$ pfu at week 10

All vaccines were administered intramuscularly. vp = virus particles. pfu = plaque forming units

### 2.1.2 THE HOST IMMUNE RESPONSE TO STANDARD THERAPY TRIAL (THIRST TRIAL)

All patients attending the John Radcliffe Hospital for standard hepatitis C virus therapy (PEG-IFN/ribavirin) irrespective of viral genotype were given a patient information sheet (Appendix 2) and invited to participate in this prospective study. Informed consent was obtained for all participants. 50 - 70 ml of blood was collected on days 0, 1, 7 and 28 of PEG-IFN/ribavirin treatment.

Samples taken prior to therapy commencing on day 0 served as each patient's own internal control. In addition, a control group of healthy volunteers was recruited comprising medical and research staff from the John Radcliffe hospital and Peter Medawar Building for Pathogen Research in Oxford.

23 participants provided blood samples for analysis; 13 patients with CHC undergoing standard therapy, and 12 healthy controls.

## 2.2 VIRAL LOAD AND FULL BLOOD COUNT DETERMINATION

Viral load and full blood count testing was performed by the John Radcliffe Hospital microbiology lab and biochemistry labs respectively. Viral kinetics were assessed using a quantitative DNA assay PCR (COBAS® AmpliPrep/COBAS® AMPLICOR HCV Test, v2.0); lower limit of detection, 15 IU/ ml.

## 2.3 PLASMA PREPARATION

At each appropriate time point, 6 ml of blood was collected in an EDTA coated tube. Samples were then centrifuged for 10 minutes at 2000 × g within 2 hours of collection. Supernatant (plasma) was then aspirated and stored at -20 $^{o}$C.

## 2.4 LYMPHOCYTE COLLECTION/FREEZING: METHOD USED FOR THIRST AND HCV003 TRIALS

### 2.4.1 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) PREPARATION

An average volume of 50 ml of blood was collected at each time point and layered onto lymphoprep in a 50 ml BD Falcon™ Conical Tube before centrifuging at 2200 rpm for 20 minutes (without brakes). Cells were removed from the buffy layer using a Pasteur pipette and washed in R10 (RPMI medium and 10% fetal calf serum). The cells were spun down again at 1800 rpm for 10 minutes with braking and the supernatant removed. 25 ml of R10 was added to the pellet and the cells were counted, and then washed again by spinning at 1600 rpm for 7 minutes.

### 2.4.2 FREEZING/THAWING PBMCS

Cells were frozen for later use in 90% FCS/ 10% DMSO solution (approximately 1 ml per 5-10 million cells) initially at -80 $^{o}$C. Frozen vials were transferred to liquid nitrogen (-180 $^{o}$C) 1 - 3 days later.

At thawing, vials were removed from liquid nitrogen storage and transferred to a 37 $^{o}$C water bath. When partially thawed, vial contents were transferred to 15 ml Falcon tubes. To reduce osmotic load and preserve cell integrity, a solution containing 1 ml CTL wash, 100 µl DNAse and 9 ml RPMI was slowly added using a Pasteur pipette over 2 - 3 minutes. The sample was then centrifuged and the supernatant discarded. The cell pellet was then resuspended in R10 media.

## 2.5 HCV SPECIFIC PEPTIDE POOLS

A set of 494 peptides, 15 amino acids (15mer) in length overlapping by 11 amino acids spanning the genome of HCV genotype 1B strain BK (sequence accession number M58335), were grouped into 10 pools (A - I, L & M, range 73 - 112 peptides/pool). Pools F-M represented the open reading frame from NS3-NS5B equivalent to the vaccine insert. These were obtained from BEI resources. Pools were used at a final concentration of 3 µg/ ml or 1 µg/ ml (each single peptide) for ELISpot assays and intracellular cytokine staining (ICS) respectively.

## 2.6 ELISPOT DETERMINATION OF IFN-γ PRODUCTION FOLLOWING HCV PEPTIDE STIMULATION

For patients enrolled in the HCV003 trial, IFN-γ ELISpot assays were performed by research assistants Anthony Brown and Rachel Townsend on freshly isolated PBMCs. The majority of fine mapping of positive pools by IFN-γ ELISpot assays was performed by myself with the assistance of Anthony Brown.

### 2.6.1 IFN-γ ELISPOT PROTOCOL

IFN-γ ELISpot assays were performed according to the manufacturer protocol (Mabtech).

**DAY -1** Millipore AIP plates were first coated with 100 µl/ well of anti-human IFN-γ antibody (5 µg/ ml in PBS) and left overnight at 4 °C.

**DAY 0** The following day, unbound antibody was washed off with PBS. Plates were then blocked with R10 (200 µl/ well) and incubated at 37 °C for 2 hours. Cells were then plated at $2 \times 10^5$ PBMCs/ well. Each of the 10 HCV specific peptide pools (final concentration 3 µg/ ml, described in Section 2.5) was then added to triplicate wells. Peptide/PBMCs were co-cultured overnight at 37 °C.

Internal controls were; i) DMSO (negative control), and ii) 2 positive controls: Concavalin A and FEC (mixed HLA class I-restricted peptides from influenza, Epstein-Barr virus and CMV).

**DAY 1** The supernatant from each well was first removed and stored at -80 °C for future analysis. Plates were then washed 7 times with PBS/Tween wash buffer using a plate washer (blotting dry between each wash). Biotinylated mouse anti-human IFN-γ (0.5 μg/ ml in PBS) was added and plates incubated at room temperature for 2 - 4 hours.

Next, plates were washed with PBS/Tween wash buffer 4 times before adding 50 μl of alkaline phosphatase antibiotin antibody (diluted 1:750 in 0.5% BSA/PBS). Plates were then incubated for another 2 hours at room temperature before washing another 4 times. 50 μl of BCIP/NBT solution was added to each well and incubated for 7 minutes before rinsing plates again. Plates were then left to dry overnight at room temperature.

**DAY 2** After drying, plates were read manually using an ELISpot plate reader (AID®). Each pool's final response was calculated by subtracting the mean DMSO response (background) from the mean response for each pool. The total NS response was calculated by summing final pool responses for pools F - M.

### 2.6.2 DEFINITION OF POSITIVE IFN-γ ELISPOT RESPONSE

A positive pool response was defined as i) a response ≥ 39 Spot Forming Units (SFU)/$10^6$ PBMCs, and ii) to exceed 3 times the background. This cut-off was established based on the mean (+3 standard deviations) of screening IFN-γ ELISpot responses in DMSO pools from 58 HCV infected patients who had previously been assessed using the same IFN-γ protocol in the Barnes/Klenerman lab.

A vaccine induced T cell response was defined as, either of; i) a new positive pool response after vaccination that was not detected at baseline, or ii) an increase in a previously positive pool response of > 30% of baseline.

Total NS response was calculated by summing pools F - M after subtracting the mean background response from each pool.

### 2.6.3 FINE MAPPING OF POSITIVE IFN-γ ELISPOT RESPONSES

Where possible, the individual peptides within positive IFN-γ ELISpot pools to which T cells were responding were identified by fine mapping. In brief, minipools were generated by dividing positive pools into groups of ~ 10 peptides, and then PBMC responses to these minipools were assessed by IFN-γ ELISpot assay. Positive minipools were then mapped to individual peptides through a similar process.

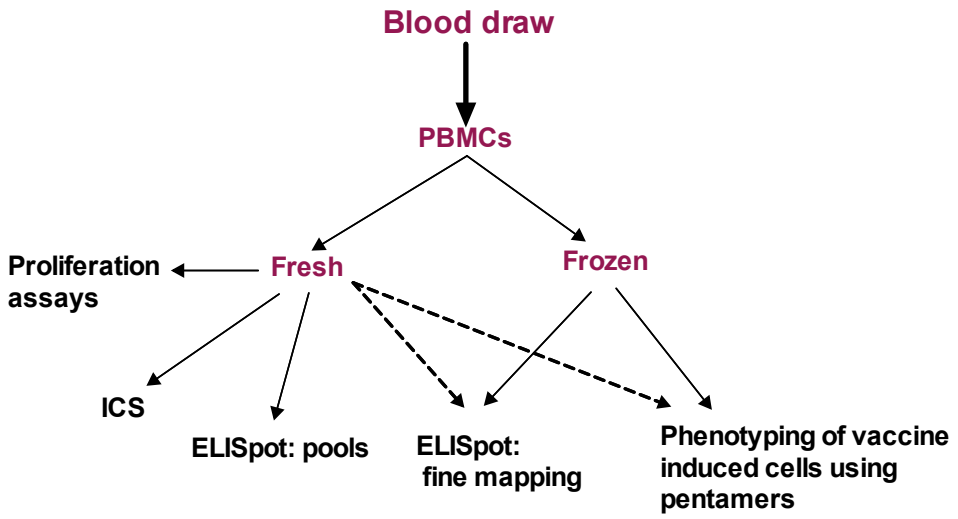
### 2.6.4 PROLIFERATION ASSAYS

This assay was performed by Anthony Brown (R.A at Barnes/Klenerman lab). Briefly, ex-vivo proliferation assays were performed on freshly isolated PBMCs plated in triplicate at $2 \times 10^5$ PBMCs per well with conventional [$^3$H]thymidine incorporation methods and antigens (1 mg/ ml) (Mikrogen). A positive response was defined as SI ≥ 3.

## 2.6.5 SUMMARY OF T CELL ASSAYS PERFORMED IN THE HCV003 TRIAL

A summary of the T cell assays performed in the HCV003 is presented in Figure 2-1

Figure 2-1 Summary of T cell assays performed in HCV003 trial




ICS= intracellular cytokine staining

## 2.7 SEQUENCING OF CIRCULATING VIRUS IN PATIENTS WITH CHRONIC HCV INFECTION

### 2.7.1 RNA EXTRACTION

Plasma was separated from whole blood by centrifugation (2000 rpm for 10 minutes) and stored at -80 °C in 500 µl aliquots. At the time of RNA extraction, thawed plasma was centrifuged (23,600 × *g* at 4 °C for 60 minutes) to pellet RNA, and then 360 µl of supernatant was removed before resuspending. Viral RNA was then extracted using QIAamp Viral RNA mini kit (Qiagen ♯42904), eluted in AVE elution buffer (50 µl) and stored at -80 °C

### 2.7.2 POLYMERASE CHAIN REACTION (PCR)

Viral regions for sequencing were selected based on the location of vaccine induced or pre-existing T cell epitopes detected in patients recruited to the HCV003 trial. These regions were sequenced using traditional Sanger sequencing.

PCR cycle conditions and primers are detailed in Table 2-4 (page 81). RNA was reverse transcribed and 1st round PCR performed using Superscript III One-Step RT-PCR (Invitrogen ♯ 12574-035), with specific primers. Nested second round internal amplification was performed using Expand High Fidelity PCR system (Roche, ♯ 1173265001). Primers were sourced from Kuntzen el al. 2007 (Kuntzen et al. 2007). Of the 16 PCR cycle conditions used, 8 had been previously designed by D.Phil. student Dr Kelly. I designed the remaining 8 sequencing protocols.

### 2.7.3 PCR PRODUCT EXTRACTION

PCR products were run on 2% agarose gels containing ethidium bromide (4 µl/ 100 ml gel) for 45 - 60 minutes depending on fragment length (at 120 V). Where single bands were present, DNA products were direct PCR purified using QIAquick PCR purification Kit (Qiagen ＃28104). When multiple bands were present, the appropriate band was gel extracted and purified using QIAquick Gel Extraction Kit (Qiagen ＃28704).

### 2.7.4 DIRECT SEQUENCING

Amplified DNA was sequenced using 2nd round internal primers together with Prism Big Dye (Applied Biosystems) on an ABI 3100 automated sequencer. Cycling conditions were: 96 °C × 1 minute, 30 cycles of 96 °C × 15 seconds, 50 °C × 10 seconds, 60 °C × 4 minutes. Sequences were analysed using 4peaks and aligned using Se-Al software (http://www.mekentosj.com/science/4peaks/ and http://tree.bio.ed.ac.uk/software/).

Table 2-4; Primer combinations and PCR conditions used to sequence autologous virus at relevant epitopes identified in HCV003 trial

| | Geno Type* | Cycle name | Primer 5'-3' | Primer name | Location *** | PCR cycle conditions | Relevant Epitopes |
|---|---|---|---|---|---|---|---|
| 1st round | 1a | 5A | GACAAAAACCAAGTGGAGGG<br>AGGACCTTCCCCAGTCC | 5af<br>5ar | 3492<br>5868 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚C × 30 seconds, 54˚C × 30 seconds, 68˚C × 2.5 minutes then final elongation at 68˚C × 10 minutes | |
| 1st round** (Alternative) | 1a | JH1 | ATCACGGCGTACGCCCAGC<br>GGGCCCTTCTGCTTGAACTGAGA | NSEx3426 F<br>4eR | 3426<br>5519 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚C × 30 seconds, 52˚C × 30 seconds, 68˚C × 2 minutes then final elongation at 68˚C × 10 minutes | |
| 2nd round | 1a | 5B | CCTACGGCAAGTTCCTTGC<br>AGCGTGRTTGTCTCAATGG | 4bf<br>4br | 4225<br>4753 | 94˚C × 2 minutes then 39 cycles of 94˚C × 30 seconds, 52˚C × 30 seconds, 72˚C × 30 seconds then final elongation at 72˚C × 5 minutes | KLSGLGINAV<br>HSKKKCDEL |
| 2nd round | 1a | 3E | ATAGCAGGGGYAGCCTGC<br>ATCCGTGGAGTGGCACTCG | 3ef<br>3er | 3803<br>4294 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚C × 30 seconds, 52˚C × 30 seconds, 68˚C × 2 minutes then final elongation at 68˚C × 10 minutes | AYAAQGYKVLVLNPS |
| 2nd round | 1a | 5C | TGTGTCACYCAGACAGTCG<br>GGGCCCTTCTGCTTGAACTGC | 4df<br>4er | 4710<br>5519 | 94˚C × 2 minutes then 39 cycles of 94˚C × 30 seconds, 57˚C × 30 seconds, 72˚C × 1 min then final elongation at 72˚C × 5 minutes | SVVIVGRIIL<br>EFWESVFTGL |
| 1st round* | 1b | JH2A | GCCGCGATGCCATCATCC<br>CATTAGAGCGTCTGTTGC | 1bGL3bF<br>1bGL4bR | 2954<br>4666 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚C × 30 seconds, 50˚C × 30 seconds, 68˚C × 2 minutes then final elongation at 68˚C × 10 minutes | |
| 2nd round | 1b | JH2B | AAGGACCATCACCACGGG<br>CATTAGAGCGTCTGTTGC | 1b4bF<br>1bGL4bR | 4206<br>4666 | 94˚C × 2 minutes then 39 cycles of 94˚C × 30 seconds, 50˚C × 30 seconds, 72˚C × 30 seconds, then final elongation at 72˚C × 5 minutes | KLSGLGINAV<br>HSKKKCDEL |
| 1st round | 1a | 6A | GCTCGCYGAGCAGTTCAAGC<br>TYGACCATGACCCGTCGC | 5bF<br>6cR | 5510<br>7560 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚C × 30 seconds, 54˚C × 30 seconds, 68˚C × 2 minutes then final elongation at 68˚C × 10 minutes | |
| 2nd round | 1a | 6C | AGGAACATGTGGAGTGGG<br>GTTYTCTGACTCAACCCTGG | 6aF<br>6aR | 6498<br>7085 | 94˚C × 2 minutes then 39 cycles of 94˚C × 30 seconds, 53˚C × 30 seconds, 72˚C × 45 seconds then final elongation at 72˚C × 5 minutes | EVDGVRLHR |
| 1st round | 1b | 6bA | GTGGAAGTGTCTCAYACG<br>ATGTTYCCGCCCATCTCCTGCCG | 1b5aF<br>1b6aR | 5445<br>7045 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚ × 30 seconds, 54˚C × 30 seconds, 68˚C × 2 minutes then final elongation at 68˚C × 10 minutes | |
| 2nd round | 1b | 6bC | CACCTGCCCATGTGGAGC<br>AGGGGTCGGTRAGCATG | 1b5eF<br>1b6eR | 6430<br>6860 | 94˚C × 2 minutes then 39 cycles of 94˚C × 30 seconds, 55˚C×30 seconds, 72˚C × 45 seconds then final elongation at 72˚C×5 minutes | EVDGVRLHR |
| 1st round | 1a | 7A | CCACATCAACTCCGTGTGG<br>TTCATCGGTTGGGGAGGAGG | 7bf<br>7er | 7952<br>9361 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚C×30 seconds, 53˚C × 30 seconds, 68˚C × 2 minutes then final elongation at 68˚C × 10 minutes | |
| 2nd round | 1a | 7C | GCCTGCTACTCCATAGAACC<br>TTCATCGGTTGGGGAGGAGG | 7ef<br>7er | 8971<br>9361 | 94˚C × 2 minutes then 39 cycles of 94˚C × 30 seconds, 55˚C × 30 seconds, 72˚C × 40 seconds then final elongation at 72˚C × 4 minutes | SQLDLSGWFVAG<br>SRARPRWFM |
| 1st round | 1b | 7bA | CGCTGYTTTGACTCAACG<br>ATTGGCCTGGAGTGTTTA | 1b7cF<br>1b 3'R | 8297<br>9400 | 50˚C × 45 minutes, 94˚C × 2 minutes, 39˚C cycles of 94˚C × 30 seconds, 53˚C × 30 seconds, 68˚C × 2 minutes then final elongation at 68˚C × 10 minutes | |
| 2nd round | 1b | 7bC | CYCACTTCTTCTCCATCC<br>ATTGGCCTGGAGTGTTTA | 1b7eF<br>1b3'ext | 8297<br>9400 | 94˚C × 2 minutes then 39 cycles of 94˚C × 30 seconds, 51˚C × 30 seconds, 72˚C × 40 seconds then final elongation at 72˚C × 4 minutes | PAASQLDLSGWFVAG<br>LSRARPRWFML |

*Primer combinations were chosen appropriate for the genotype of autologous circulating virus. **This option was used in cases were 1st round 5A was unsuccessful. ***Location is based on nucleotide number.

## 2.8 ANTIBODY STAINING FOR FLOW CYTOMETRY ASSESSMENT OF CELL PHENOTYPE

### 2.8.1 DETERMINING APPROPRIATE ANTIBODY CONCENTRATION

Antibody clones that had not previously been tested by other researchers in the Barnes/Klenerman laboratory were first titrated to assess best concentration. See Appendix 4 (page 282) for an example FACS plot and histogram demonstrating this process.

### 2.8.2 CELL PREPARATION

After thawing, cells were resuspended in 50 µl R10 on 96 well plates with 1 x $10^6$ cells/well and rested overnight in a 37 $^oC$ incubator.

### 2.8.3 STIMULATION OF CELLS FOR FUNCTIONAL ASSESSMENT OF CYTOKINE PRODUCTION

#### *HCV003 trial*

Fresh PBMCs were plated into 96 well plates at 1 × $10^6$ cells/well in 100 µl RH10. Cells were then stimulated with either pooled peptides (pool F+G+H = NS3/4, I+L+M = NS5A/B) or PMA at 50 and 500 ng/ml respectively. One well/sample was left unstimulated with DMSO at 5 ng/ml. After 2 - 4 hours stimulation, Brefeldin A was added (10 µl/ml) and cells were incubated overnight at 37 °C before staining. This assay was performed by Leo Swadling.

#### *THIRST trial*

Cells that were to be assessed for cytokine production were first stimulated with PMA and ionomycin at 250 ng/ml and 500 ng/ml respectively, and incubated at 37 °C. After 1 hour,

Brefeldin A was added at 1 µg/ml to the functional and control wells. 5 hours later cells were washed twice with 200 µl PBS before staining.

## 2.8.4 SURFACE STAINING OF PBMCS FOR FLOW CYTOMETRY

Next, cells were resuspended in 50 µl PBS and incubated for 45 minutes at 4 $^{o}$C together with the appropriate surface marker antibody combinations for each flow cytometry panel (see Table 2-5). Unlike other antibodies, CD4 (on Qdot605) was not added prior to fixation because fixation can cause breakdown of the Qdot605 fluorochrome. After staining, cells were washed twice in PBS to remove unbound antibody. Those panels for which no intracellular staining was required were fixed with 2% formaldehyde for 10 minutes, washed twice in PBS and then stained with CD4, CD3 and CD8 for 25 minutes.

## 2.8.5 INTRACELLULAR STAINING FOR FUNCTIONAL AND ICS FLOW CYTOMETRY PANELS

### *Fixation/Permeabilisation*

Following surface staining, cells for functional and ICS staining were resuspended in 100 µl of FoxP3 fix/perm (eBioscience ♯00-5523-00) and incubated in the dark for 40 minutes at 4 $^{0}$C. Cells were then washed twice in Foxp3 perm buffer and resuspended in 50 µl Foxp3 Perm Buffer.

### *ICS Staining*

Appropriate ICS antibody combinations for each panel were then added (Table 2-5, page 85) and stained for 50 minutes at room temperature. Cells were then washed twice with PBS and kept on ice until FACS analysis.

### *HLA Class I pentamer staining for HCV003 trial*

I performed this assay with the guidance of fellow D.Phil student, Leo Swadling. HLA*0201 matched pentamers (epitope $NS_{1406}$) labeled with PE were obtained from Proimmune Ltd. 500,000 cells/well were first suspended in 50 µl of PBS and then stained for 20 minutes at 37 °C with pentamer at a concentration of 1/50. Unbound pentamer was removed by washing (3 times by centrifuging for 7 minutes at 1400 × *g*, removing supernatant and resuspending in PBS). Next cells were stained with live/dead antibody for 10 minutes before washing and then fixing with 1% formaldehyde in PBS. Cells were next stained with either panel 1 or permeabilised (using Foxp3 Perm buffer 1:10) and stained with panel 2. (Table 2-6)

## 2.8.6 FACS ANALYSIS

FACS analysis was carried out on an LSRII FACS caliber machine.

### *Functional response to PMA/Ionomycin stimulation in the THIRST study*

To determine true percentages of cells responding to PMA/ionomycin stimulation, the percentage of cells producing the relevant cytokine in an unstimulated control sample was subtracted from each sample.

**Table 2-5; FACS panels for THIRST trial**

| **Fluorochrome** | **FITC** | **APC** | **PerCP/ Cy5.5** | **PE** | **PEcy7** | **Alx700** | **APC/Cy7** | **Pacific Orange** | **QD605** | **V450** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Panels (1-5)** | | | | | | | | | | |
| **1. Activation** | | | | | | | | | | |
| **Antibody** | **CD69** | **HLA-DR** | | **CD38** | | | | | | |
| Concentration | 1/50 | 1/50 | | 1/50 | | | | | | |
| Clone/lot | FN50 | MLHDR05 | | HB7 | | | | | | |
| Company | Ebioscience | Invitrogen | | Ebioscience | | | | | | |
| **2. Differentiation** | | | | | | | | | | |
| **Antibody** | **CD57** | **CD45RA** | | **CCR7** | **CD62L** | | | | | |
| Concentration | 1/50 | 1/100 | | 1/20 | 1/25 | | | | | |
| Clone/lot | Lot59924 | Lot526786 | | 3D12 | DREG-56 | | | | | |
| Company | BD | Abcam | | Ebioscience | Biolegend | | | | | |
| **3. Homing/ Subsets** | | | | | | | **Live/Dead** | **CD3** | **CD4** | **CD8** |
| **Antibody** | **CD16** | **CD161** | **CCR6** | **CXCR3** | **CD56** | **CD19** | | | | |
| Concentration | 1/50 | 1/100 | 1/50 | 1/20 | 1/25 | 1/25 | | | | |
| Clone/lot | CB16 | 191B8 | TG7 | 49801 | B159 | LT19 | | | | |
| Company | eBioscience | MACS | Biolegend | RND | BD | AbD | | | | |
| **4. ICS** | | | | | | | | | | |
| **Antibody** | **CD127** | **CD25** | | **Perforin** | **FoxP3** | **Granzyme B** | | | | |
| Concentration | 1/50 | 1/50 | | 1/100 | 1/50 | 1/50 | | | | |
| Clone/lot | MB15-18C9 | BC96 | | δG9 | PCH101 | GB11 | | | | |
| Company | Miltenyi | Biolegend | | BD | Ebioscience | BD | | | | |
| **5. Functional** | | | | | | | | | | |
| **Antibody** | **CD56** | **CD161** | **IL-2** | **IL-17** | **TNF-α** | **IFN-γ** | | | | |
| Concentration | 1/50 | 1/100 | 1/25 | 1/25 | 1/25 | 1/50 | 1/250 | 1/50 | 1/200 | 1/100 |
| Clone/lot | MEM188 | Miltenyi | MQ1-17H12 | 64dec17 | Mab11 | B27 | Invitrogen | UCHT1 | S3.5 | RPA-T8 |
| Company | Ebioscience | Lot 5101020141 | BD | Ebioscience | BD | BD | (Near I/R) | Invitrogen | Invitrogen | BD |

**ICS= Intracellular staining.**

**Table 2-6; FACS panels for HLA*0201 matched pentamer reactive T cells in HCV003 trial**

| Fluorochrome | FITC | APC | PerCP/Cy5.5 | PE | PEcy7 | Alx700 | Alexa750 | Pacific Orange | Pacific Blue |
|---|---|---|---|---|---|---|---|---|---|
| **Panels (1-2)** | | | | | | | | | |
| **1. Act/diff Antibody** | **CD45RA** | **CD127** | **CD38** | **Pentamer** | **CCR7** | **HLA-DR** | **Live/Dead** | **CD3** | **CD8** |
| Concentration | 1/50 | 1/50 | 1/50 | | 1/50 | 1/50 | | | |
| Clone/lot | H1100 | MB15-18C9 | HIT2 | | 3D12 | G46-6 | | | |
| Company | BD | Miltenyi | Biolegend | | BD | BD | | | |
| **2. Cytotoxicity Antibody** | **Perforin** | **CD161** | **Granzyme A** | | **PD-1** | **Granzyme B** | | | |
| Concentration | 1/50 | 1/100 | 1/100 | | 1/50 | 1/50 | 1/250 | 1/50 | 1/200 |
| Clone/lot | dG9 | 191B8 | CB9 | | EH12.1 | GB11 | Invitrogen | UCHT1 | RPA-T8 |
| Company | BD | Miltenyi | Biolegend | | BD | BD | (Near I/R) | Invitrogen | BD |

**These 2 panels were designed by D.Phil student Leo Swadling originally for assessment of vaccine induced T cells in healthy volunteer HCV003 trial participants**

**Table 2-7; FACS panel for murine experiments**

| Fluorochrome | FITC | PerCP/ Cy5.5 | PE | PEcy7 | Alx700 | APC/Cy7 | Viogreen | Efluor450 |
|---|---|---|---|---|---|---|---|---|
| **Panel** | | | | | | | | |
| **Antibody** | **CCR9** | **CXCR3** | **B7 integrin** | **CCR6** | **CD4** | **Live/dead** | **CD8** | **CD3** |
| Concentration | 1/100 | 1/50 | 1/50 | 1/100 | 1/50 | 1/250 | 1/50 | 1/100 |
| Clone/lot | CW-1.2 | CXCR3-173 | FIB27 | 29-2117 | GK1.5 | Invitrogen | 53-6.7 | 17A2 |
| Company | Biolegend | eBioscience | Biolegend | Biolegend | Biolegend | (Near I/R) | Miltenyi | eBioscience |

## 2.9 IL-28 GENOTYPING WITH REAL-TIME qPCR

IL28 genotype was determined by testing for the polymorphism rs8099917. The possible genotypes for this polymorphism are GG, GT and TT. Those patients with genotype 1 HCV infection who are homozyogous for the TT allele have a better response to standard HCV therapy and are more likely to clear virus in the setting of acute infection. I performed IL-28 genotyping of the 13 patients enrolled in the THIRST trial. Rachel Townsend (Research Assistant) performed this assay for patients enrolled in the HCV003 trial.

### 2.9.1 DNA EXTRACTION

| Reagent/Material | DESCRIPTION |
|---|---|
| RBC lysis solution | Qiagen Gentra Puregene kit (＃158389) |
| Cell lysis solution | |
| Protein precipitation solution | |
| DNA hydration solution | |
| RNase A solution | |
| Isopropanol | |
| 70% ETOH | |

DNA was purified from whole blood using the Qiagen Gentra Puregene kit (1068994) according to the manufacturer's instructions. Briefly, 3 ml of whole blood was added to 9 ml of RBC lysis solution in a 15 ml Falcon tube and mixed (inverted 10 times). After incubation for 5 minutes, sample was centrifuged for 2 minutes at 2000 × g to pellet white blood cells. The supernatant was then discarded leaving approximately 200 µl of residual fluid and the pellet resuspended by vortexing. Next 3 ml of Cell Lysis Solution was added and mixed by vortexing. Samples were then stored at room temperature (in Cell Lysis Solution) until collective analysis of IL-28 at a later date.

At the time of IL-28 genotyping, 1 ml of Protein Precipitation Solution was added to the sample and vortexed before centrifuging for 5 minutes at 2000 × *g*. Next, the supernatant was poured into a fresh Falcon tube containing 3 ml of isopropanol (leaving the protein

pellet behind) and mixed by inverting 50 until the DNA was visible as threads. The sample was then pelleted by centrifuging for 3 minutes at 2000 × *g*, and the supernatant discarded. 3 ml of 70% ETOH was added and the sample inverted several times to wash the DNA before centrifuging for 1 minute at 2000 × *g* and again discarding the supernatant. Finally, 300 µl of DNA Hydration Solution was added and vortexed before incubating for 1 hour at 65 °C to dissolve the DNA. Samples were then incubated at room temperature overnight with gentle agitation before centrifuging and transferring to a storage tube.

## 2.9.2 IL-28 GENOTYPING

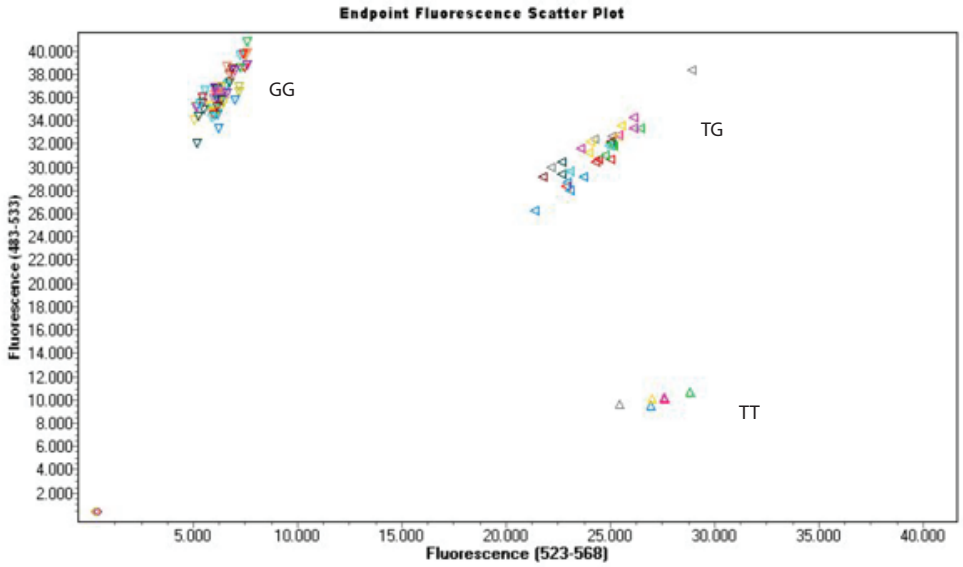
| Reagent/Material | Description |
|---|---|
| SNP genotyping mix containing:<br><br>Probe 1 sequence (bound to VIC® dye)<br><br>Probe 2 sequence (bound to FAM® dye) | (SNP id rs809917)<br><br>TTTTGTTTTCCTTTCTGTGAGCAATGTCACCCAAATTGG AACCATGCTGTA<br><br>TTTTGTTTTCCTTTCTGTGAGCAATTTCACCCAAATTGG AACCATGCTGTA |
| Master Mix | Applied Biosystems (#11710096) |
| DNA | Patient sample, and positive/negative controls |
| Distilled $H_20$ | |

Genotyping was performed using the ABI TaqMan allelic discrimination kit from Applied Biosystems (#11710096). DNA quantity and quality was first assessed using a Nanodrop® spectrophotometer. Next, working on ice and under low level lighting (the SNP genotyping mix and master mix are sensitive to light) the 96 well plate layout was first determined including positive (allele G, GT & T) and negative controls. All samples were run in triplicate.

In the clean hood: 13.75 µl of Master Mix and SNP genotyping mix were added to each well.

In the dirty hood: diluted DNA (1 – 20 ng) to each well (equal amounts of DNA in all wells is required). Sterile distilled water was added in place of DNA to negative control wells and the plate covered with a plate sealer before spinning at 1000 rpm for 20 – 30 seconds to ensure all liquid was at the bottom of the well. The plate was then run on the Light Cycler real-time PCR machine (LC480) with an output as shown in Figure 2-2.

Figure 2-2 Fluorescence Scatter Plot for IL-28 genotyping generated by Light Cycler real-time PCR




Each coloured triangle represents an individual patient. X-axis: Fluorescence for allele T. Y-axis: fluorescence for allele G. ie. those samples clustered in the top left of the figure are G/G homozygous (as indicated)

## 2.10 ASSESSMENT OF CXCL10 PRODUCTION BY HEPATOCYTES IN-VITRO FOLLOWING IFN-α STIMULATION

I was kindly assisted by Dr Narayan Ramamurthy a post-doctoral fellow at the Barnes/Klenerman lab in this set of experiments (through to qPCR).

### 2.10.1 CELL STIMULATION AND RNA EXTRACTION PROTOCOL

| Reagent/Material | Description |
|---|---|
| Huh7.5 Cells | Cells kindly provided by Dr N. Ramamurthy |
| IFN-α | PBL Biomedical Laboratories via R&D (＃11105-1) |
| RIPA buffer (+protease inhibitor) | Thermo scientific (＃89901) |
| DMEM cell culture media | Invitrogen (high glucose with glutamax 4.5 g) |
| RNA extraction minikit | Qiagen (#74106) |

Cells from the hepatoma line, Huh7.5 were seeded into 6 well plates, suspended in 2 ml DMEM and rested overnight. The following day cells were stimulated with IFN-α at concentrations of; 0 IU/ml (control) and 1000 IU/ml. RNA was extracted using Qiagen RNA extraction minikit (#74106). Briefly cells were lysed using 350 µl of RLT buffer (with RNAase inhibitors) after 30 minutes, 60 minutes, 2 hours and 24 hours incubation with IFN-α. RNA was extracted following manufacturers protocol using Qiagen minikit. The extracted RNA was then treated with DNAse and RNA was then stored at -80 °C.

## 2.11 ASSESSMENT OF CXCL10 PRODUCTION BY qPCR

### 2.11.1 REVERSE TRANSCRIPTION TO cDNA

Reverse transcription was performed using the Superscript III Reverse Transcriptase Kit from Invitrogen (#18080-085).

| Reagent/Material | DESCRIPTION |
|---|---|
| 5xFirst Strand Buffer | Invitrogen #18080-085 |
| 0.1 M DTT | Invitrogen #18080-085 |
| SuperScript III RT | Invitrogen #18080-085 |
| Oligo (dT)(50 µM) | Invitrogen #18418-020 |
| Hexamers (random primers) | Invitrogen #48190-011 |
| 10 mM dNTP mixture | Invitrogen #18427-013 |
| RNase OUT | Recombinant RNase inhibitor 40 IU/µL<br>Invitrogen #10777-019 |
| MilliQ water | Invitrogen |

Extracted RNA was quantified by using a Nanodrop® spectrophotometer and 100 ng of RNA/reaction aliquoted into Eppendorf tubes. Each sample was then topped up to a total volume of 5 µl with MilliQ water. Next 1 µl oligo(dT), 0.1 µl random primers, 1 µl 10 mM dNTPs and 5.9 µl $dH_20$ was added to each sample (via a mastermix). RNA was denatured by heating samples to 65 °C for 5 minutes before cooling to 4 °C in the PCR machine.

To each sample was then added 7 µl containing: 4 µl 5x first strand buffer, 1 µl 0.1 M DTT, 1 µl RNase OUT and 1 µl of Superscript III was added (via a mastermix). Samples were run in the PCR machine at: 25 °C for 5 minutes, 50 °C for 60 minutes, 70 °C for 15 minutes and then 4 °C for ever. cDNA was stored at -20 °C until qPCR.

## 2.11.2 QUANTITATIVE PCR USING ROCHE LIGHT CYCLER 480

| Reagent/Material | DESCRIPTION |
|---|---|
| Roche Light Cycler 480 SYBR Green Master mix | Roche ♯04707516001 |
| CXCL10 Primers 10 µg/mL | F: 5'-- GAAAGCAGTTAGCAAGGAAAGGT--3'<br>R: 5'-- GACATATACTCCATGTAGGGAAGTGA--3'<br>(Designed using Roche Universal ProbeLibrary. CCL10 accession No. NM_001565) |
| CXCL10 Probe | No. 34 (Roche ♯04687971001) |
| GAPDH primers | F: 5' --AGCCACATCGCTCAGACAC--3'<br>R: 5' --GCCCAATACGACCAAATCC--3'<br>(Designed using Roche Universal Probe Library. GAPDH accession No. NM_002046) |
| GAPDH probe 60 | No. 60 (Roche ♯04688589001) |
| cDNA | |
| Roche Light Cycler white plate and cover | |

This was performed using a Roche kit (♯04707516001). cDNA was thawed and diluted 1/30 in $dH_2O$ before adding 5 µl to each well. Mastermixes for each gene of interest containing: 10 µl of SYBR Green MasterMix, 1 µl of Forward Primer, 1 µl of Reverser Primer, 0.3 µl Probe and 2.7 µl of $dH_2O$ were prepared and then added to each reaction well (15 µl total). qPCR was performed on the LightCycler machine: 95 °C for 5 minutes, then 55 °C cycles of: 94 °C for 10 seconds, 58 °C for 30 seconds and 72 °C for 10 seconds, followed by 40 °C for 10 seconds.

## 2.12 ELISA ASSAY FOR CXCL10

As an example of methodology, the ELISA assay for CXCL10 is described below. This kit is commercially available (R&Dsystems, #DIP100). The same process was used to determine CXCL-11 (CXCL11) using the commercially available kit (R&Dsystems, #DCX110).

| Reagent/Material | Description |
|---|---|
| CXCL10 Microplate | 96 well polystyrene microplate coated with a mouse monoclonal antibody against CXCL10. |
| CXCL10 Standard | 5 ng/vial of recombinant human CXCL10 in a buffer |
| CXCL10 Conjugate | 21 ml/vial of polyclonal antibody against CXCL10 conjugated to horseradish peroxidase |
| Assay Diluent RD1-56 | 17 ml/vial of a buffered protein base |
| Calibrator Diluent RD6Q | 21 ml/vial of buffered animal serum with preservatives. |
| Wash Buffer Concentrate | 21 ml/vial of a 25-fold concentrated solution of buffered surfactant with preservatives. |
| Color Reagent A | 12 ml/vial of stabilized hydrogen peroxide. |
| Color Reagent B | 12 ml/vial of stabilized chromogen (tetramethylbenzidine). |
| Stop Solution | 6 ml/vial of 2N sulfuric acid. |

### 2.12.1 REAGENT PREPARATION

**Substrate Solution** – 15 minutes before use Color Reagents A and B were mixed together in equal volumes and the resultant solution covered to protect from light. 200 µl of the resultant mixture was added to each well.

**CXCL10 Standard** - CXCL10 Standard was reconstituted with 1.0 ml of distilled water to produce a stock solution of 5000 pg/ml. Complete reconstitution of CXCL10 Standard was ensured by thorough mixing and then the standard was rested for a 20 minutes with gentle agitation prior to making dilutions.

**Dilution series** - 900 µl of Calibrator Diluent RD6Q was pipetted into the first Falcon (polypropylene tube) labeled 500 pg/ml. 500 µl of Calibrator Diluent RD6Q was then pipetted into the remaining tubes. CXCL10 stock solution was then added to produce a dilution series.

## 2.12.2 ELISA ASSAY PROCEDURE

75 µl of Assay Diluent RD1-56 was added to each well followed by 75 µl of Standard, control, or undiluted sample. Each sample was assayed in duplicate. The plate was then covered and incubated for 2 hours at room temperature. After incubation the wells were aspirated and washed four times using Wash Buffer (400 µl) ensuring complete removal of liquid at each wash by blotting against clean paper toweling.

Next, 200 µl of CXCL10 Conjugate was added to each well and incubated for 2 hours at room temperature. The plate was again washed four times. Next 200 µl of Substrate Solution to each well and then incubated for 30 minutes at room temperature (protected from light). 50 µl of Stop Solution was then added to each well and plate gently tapped to ensure thorough mixing. Finally, the optical density of each well was evaluated (within 30 minutes), using a microplate reader set to 450 nm.

Due to results in excess of 500 pg/ml (above maximum standard) in the first assay, a second assay was performed using plasma diluted 1/10 with calibrator diluent RD5K.

## 2.13 MURINE EXPERIMENTS

C57/BL6 mice were obtained from Harian Laboratories. Mice were all female and 6 weeks of age at the time of use. Dr Arturo Reyes-Sandal assisted in this part of the project - specifically, in my presence he; i) handled the live mice and performed tagging and injecting, and ii) sacrificed the mice and extracted their organs. After this, at the Jenner Institute we worked together isolating lymphocytes from spleen, liver and lymph nodes and processed whole blood as outlined below. Samples were then transferred to the Peter Medawar Building for Pathogen Research where I prepared them for FACS analysis according to the method outlined in Section 2.6. Lymphocytes and whole blood were stained using the panel in Table 2-7 (page 86).

A summary of the experiment protocol is given in Figure 2-3. Mouse IFN- α was obtained from Miltenyi Biotech (＃130-093-131).

Figure 2-3; Murine experiment protocol summary

| | **Group 1 (IFN-α group)** | **Group 2 (Control group)** |
|---|---|---|
| **Day 0** | i) Inject single dose of IFN-α 100,000 IU/mouse (sc)<br><br>ii) Take blood for baseline lymphocyte count (from tail vein) | i) Inject PBS 100 µl/mouse (sc)<br><br>ii) Take blood for baseline lymphoctye count (from tail vein) |
| **Day 1** | i) Take blood for lymphocyte count and FACS staining<br>ii) Sacrifice mice and harvest organs: Liver, spleen, pre-auricular lymph nodes<br>iii) Isolate lymphocytes from organs for FACs staining | |
| sc = subcutaneously | | |

### 2.13.1 LYMPHOCYTE EXTRACTION FOR MOUSE EXPERIMENT

| Reagent/Material | Description |
|---|---|
| Percoll | GE healthcare (#17-0891-01) |
| RPMI 5% FCS + Pen/Strep | |
| FACS buffer | PBS, 2% FCS, 0.1% NaN3, 5 mM EDTA |
| ACK lysis buffer | (red cell lysis buffer) Invitrogen #A10492-01 |
| PBS | Phosphate buffered saline |
| 96 well plates | Round bottom |
| 40 µm cell strainer | |
| Glass pipette | VWR International. (#612/1701) |

#### *Spleen*

Isolated spleens were first transferred to 1 ml PBS in an Eppendorf tube. A single cell suspension was then prepared by mechanical disruption of each spleen through a 40 µm cell strainer using a syringe plunger. Each spleen was first crushed using the hard end of syringe in a plate. Crushed spleens were then transferred to a 50 ml falcon capped with cell strainer and pushed through the strainer using a soft black plunger, together with PBS for flushing. The suspension collected in the Falcon tube was then centrifuged for 5 minutes at 1200 rpm. The supernatant was then discarded and pellet resuspended in 1 ml ACK buffer and incubated for 1 minute at RT. 10 ml of PBS was then added to wash the cells. The cells were then centrifuged for 5 minutes at 1200 rpm. The supernatant was discarded and pellet resuspended in 3 ml PBS before surface staining for FACS.

#### *Liver*

Before organ extraction, each liver was perfused by injecting 10 ml PBS into the portal vein (until the liver was light brown in colour). Murine livers were then extracted and transferred individually to 15 ml Falcon tubes containing 10 ml PBS. A single cell suspension was prepared by mechanical disruption of the livers with a 40 µm cell strainer, a syringe plunger and PBS for flushing. The cell suspension was collected in a Falcon tube and then centrifuged for 5 minutes at 1200 rpm. Supernatant was then discarded and the

remaining pellet resuspended in 5 ml of 30% Percoll in PBS in a 15 ml FALCON tube. Next, 3 ml of 70% Percoll in PBS was carefully underlayed using an open topped glass pipette. After this, the sample was centrifuged at 2000 rpm for 25 minutes (without brake). Fat and debris from the top layer of the Falcon was then removed using a Pasteur pipette leaving about 0.5 cm of liquid above the interphase. Next, the interphase was collected using a Pasteur pipette and transferred to a new Falcon tube filled with PBS. The sample was then centrifuged for 5 minutes at 1500 rpm, supernatant discarded and the remaining pellet was resuspended in 1 ml ACK buffer. After 1 minute incubation at room temperature, cells were washed with 10 ml of PBS. The sample was once again centrifuged for 5 minutes at 1500 rpm, supernatant discarded and the remaining pellet was resuspended in 2 ml of PBS awaiting FACS staining.

#### *Lymph nodes*

The 2 pre-auricular lymph nodes from each mouse were combined and mechanically disrupted on a plate using a 22 gauge needle before suspending them in 20 µl PBS. The resuspended sample was then passed through a 40 µm filter and collected in an Eppendorf tube before FACS staining and quantification.

#### *Blood*

150 - 250 µl of blood was collected on day 0 and day 1 from each murine tail vein (after warming) in an Eppendorf tube containing 250 µl of EDTA. Next the Eppendorf tube was filled with ACK buffer and vortexed. The suspension was then incubated until transparent (30-60 seconds) and centrifuged at 4000 rpm for 4 minutes. The supernatant was then discarded and sample spun again briefly, prior to pipetting off the remaining supernatant. The pellet was then resuspended in 200 µl of PBS, ready for counting and FACS staining.

### 2.13.2 LYMPHOCYTE QUANTIFICATION

Lymphocytes in the mouse experiments were quantified using a CASY® counter.

## 2.14 STATISTICAL METHODS

Data were analysed using GraphPad prism software (Version 6.0a). Results were assumed to be non-Gaussian in distribution, and non-parametric tests were therefore used throughout. Values presented are presented as medians unless otherwise indicated. Paired comparisons were made using the Wilcoxon-signed rank test. For unpaired analyses, the Mann-Whitney U test was used. 2 tailed p values were used (unless otherwise indicated) and a p value of $< 0.05$ was considered statistically significantly.

# Chapter 3 Therapeutic vaccination for hepatitis C virus infection

## 3.1 PRIMARY HYPOTHESIS

Prime/boost therapeutic vaccination with ChAd3-NSmut/MVA-NSmut can safely induce T cells in patients with chronic HCV infection.

## 3.2 PRIMARY OBJECTIVES

1. To determine the safety of administering ChAd3-NSmut and MVA-NSmut vaccination in patients with chronic HCV infection
2. To determine whether HCV specific T cell responses can be induced by ChAd3-NSmut and MVA-NSmut vaccination in patients with chronic HCV infection, either alone or during concurrent PEG-IFN/ribavirin therapy.

## 3.3 KEY QUESTIONS ARISING FROM PRIMARY OBJECTIVES

1. Is it safe to vaccinate patients who have chronic HCV infection with ChAd3-NSmut and MVA-NSmut?

2. Prior to vaccination, can T cells that recognise HCV non-structural peptides be detected in HCV infected trial participants, and if so, what do they target?

3. Does ChAd3-NSmut and MVA-NSmut vaccination successfully induce T cells in HCV infected patients?

4. If T cells can be induced by vaccination, do they effectively target circulating virus?

## 3.4 CLINICAL PARAMETERS

### 3.4.1 BASELINE DEMOGRAPHICS

A summary of the baseline demographics of patients enrolled in the HCV003 trial is presented in Table 3-1. Of the patients enrolled, the median age was 49 years (range 35-59), 10/13 were male and the median duration of HCV infection was 20 years. All patients were Caucasian, and all those in the PEG-IFN/ribavirin treatment arm (Arm B) possessed a favourable IL-28 genotype (TT). Liver biopsies within 2 years of enrollment were available for 12/13 patients, and for these patients Ishak fibrosis scores ranged between 1-3/6 (where ≥5 = cirrhosis). The remaining patient (who had not been biopsied) had a Fibroscan® result of 5.4 Kpa at enrollment consistent with being non-cirrhotic. At enrollment, there was a wide range in circulating viral load (42,000 - 33.7 × $10^6$ IU/ml) and 9/13 patients were infected with genotype 1a HCV.

**Table 3-1; Demographics of HCV infected patients enrolled in HCV003 trial**

| Patient | Group | Age (yrs) | Sex | Ethnicity | HCV Geno | Fibrosis score | Duration of infection (yrs) | Baseline viral load (IU/ml) | IL-28 | HLA-A | HLA-B | HLA-C | HLA-DR | HLA-DQ | RVR | EVR | EOT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 357 | B1 | 58 | M | Caucasian | 1a | 5.4Kpa* | 14 | $33.7 \times 10^6$ | TT | 02/30 | 13/62 | 06/10 | 04/07 | 02/08 | No | No | P |
| 361 | B1 | 49 | M | Caucasian | 1a | F2/6 | 25 | $15.4 \times 10^6$ | TT | n/a | n/a | n/a | n/a | n/a | No | w/d | w/d[1] |
| 362 | B1 | 43 | M | Caucasian | 1a | F2/6 | 24 | 32,000 | TT | 29/01 | 08/44 | 16/07 | 01/07/ 53 | 02/05 | Yes | Yes | P |
| 365 | B1 | 30 | M | Caucasian | 1b | F2/6 | 10 | $2.2 \times 10^6$ | TT | n/a | n/a | n/a | n/a | n/a | No | n/a | w/d[2] |
| 350 | B2 | 56 | M | Caucasian | 1a | F1/6 | >20 | $1.8 \times 10^6$ | TT | 26/24 | 51/35 | 15/04 | 04/09 | 09/08 | Yes | Yes | Neg |
| 351 | B2 | 38 | M | Caucasian | 1b | F2/6 | 22 | $2.7 \times 10^6$ | TT | 02 | 36/50 | 06 |  |  | Yes | Yes | Neg |
| 352 | B2 | 52 | M | Caucasian | 1b | F1/6 | 6 | $9.4 \times 10^6$ | TT | 03 | 07 | 07 | 01/15/ 51 | 5/6 | No | Yes | P |
| 354 | B2 | 51 | M | Caucasian | 1a | F2/6 | 20 | $3.2 \times 10^6$ | TT | 02/01 | 62/08 | 09/07 | 52/53 | 02/08 | No | No | Positive |
| 356 | B2 | 38 | M | Caucasian | 1b | F2/6 | 10 | 173,764 | TT | 66/11 | 41/35 | 17/04 | 13 | 06 | Yes | Yes | w/d[3] |
| 355 | C1 | 59 | M | Caucasian | 1a | F3/6 | 40 | 467,000 | n/a | 02/03 | 27/65 | 08/02 | 52 | 07/06 | n/a | n/a | n/a |
| 358 | C1 | 49 | F | Caucasian | 1a | F2/6 | 27 | 42,000 | n/a | 68/03 | 44/60 | 16/10 | 7/13/52 /53 | 02/06 | n/a | n/a | n/a |
| 359 | C1 | 35 | M | Caucasian | 1a | F2/6 | 5 | 237,000 | n/a | 66/01 | 41/08 | 07/17 | 52/53 | 02 | n/a | n/a | n/a |
| 360 | C1 | 51 | F | Caucasian | 1a | F1/6 | >25 | $21.7 \times 10^6$ | n/a | 02/24 | 44/35 | 09/05 | 04/11/ 52/53 | 07 | n/a | n/a | n/a |

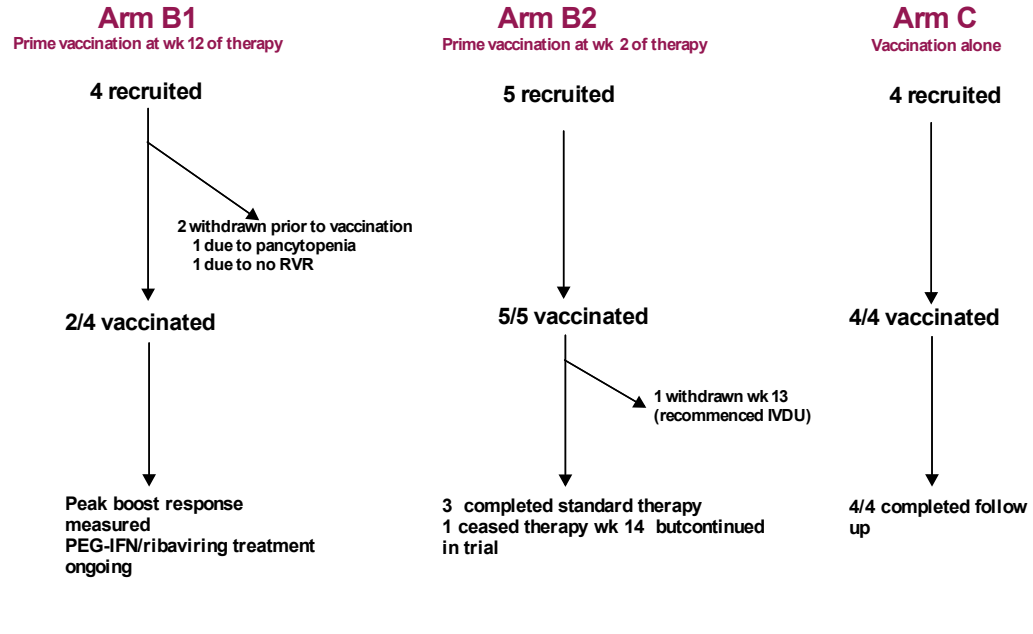
RVR= rapid virological response (viral load <50 IU/ml at wk 4). EVR = early virological response (viral load undetectable at week 12). M=male. F=female. Geno=genotype. EOT= end of treatment. P=pending, n/a = not applicable w/d = withdrawn from study. [1]w/d due to pancytopenia, [2]w/d due to failure to achieve an RVR, [3]w/d due to loss to f/up. *based on fibroscan (not biopsy). All fibrosis scores relate to liver biopsies taken within 2 years of study enrolment and are graded by Ishak fibrosis score. Where genotype subtype was not assessed, genotype 1 is listed. IL28 genotype is based on SNP rs88099917, T=protective allele.

### 3.4.2 CLINICAL PROGRESS DURING PEG-IFN/RIBAVIRIN THERAPY

A summary of recruitment data is presented in Figure 3-1. Of the HCV infected patients enrolled in the study, 11/13 completed prime (ChAd3-NSmut) and boost (MVA-NSmut) vaccination. Both patients who did not complete vaccination were enrolled in Arm B1 (prime vaccination after 12 weeks therapy) and were withdrawn prior to priming – 1 of these patients (Pt. 361) stopped PEG-IFN/ribavirin therapy after 8 weeks due to severe PEG-IFN induced pancytopenia. Another patient (Pt. 365) withdrew study consent 4 weeks into therapy when they failed to reach an RVR.

In Arm B2 (prime vaccine after 2 weeks therapy), 1 patient (Pt. 354) ceased PEG-IFN/ribavirin due to a failure to achieve an EVR after 14 weeks, but continued giving blood for immunological assessment. Another patient (Pt. 356) recommenced intravenous drug use 2 weeks after receiving boost vaccination, and withdrew consent for ongoing trial participation.

Figure 3-1; Recruitment and clinical progress of patients enrolled in HCV003 study



## 3.5 KEY QUESTION 1 - Is it safe to vaccinate patients who have chronic HCV infection with ChAd3-NSmut and MVA-NSmut?

No serious adverse events (SAEs) occurred during the course of the study. Summary safety data is presented in graphical form in Figure 3-2. Only AEs with possible, probable or definite relationships to vaccination are presented.

There was no significant change in liver biochemistry (ALT) after vaccination in any patient to suggest hepatic immunopathology (Appendix 5). For patient 357, a transient flare in ALT (peak 129 IU/ml) that occurred 2.5 months after boost vaccination was attributed to an alcohol binge.

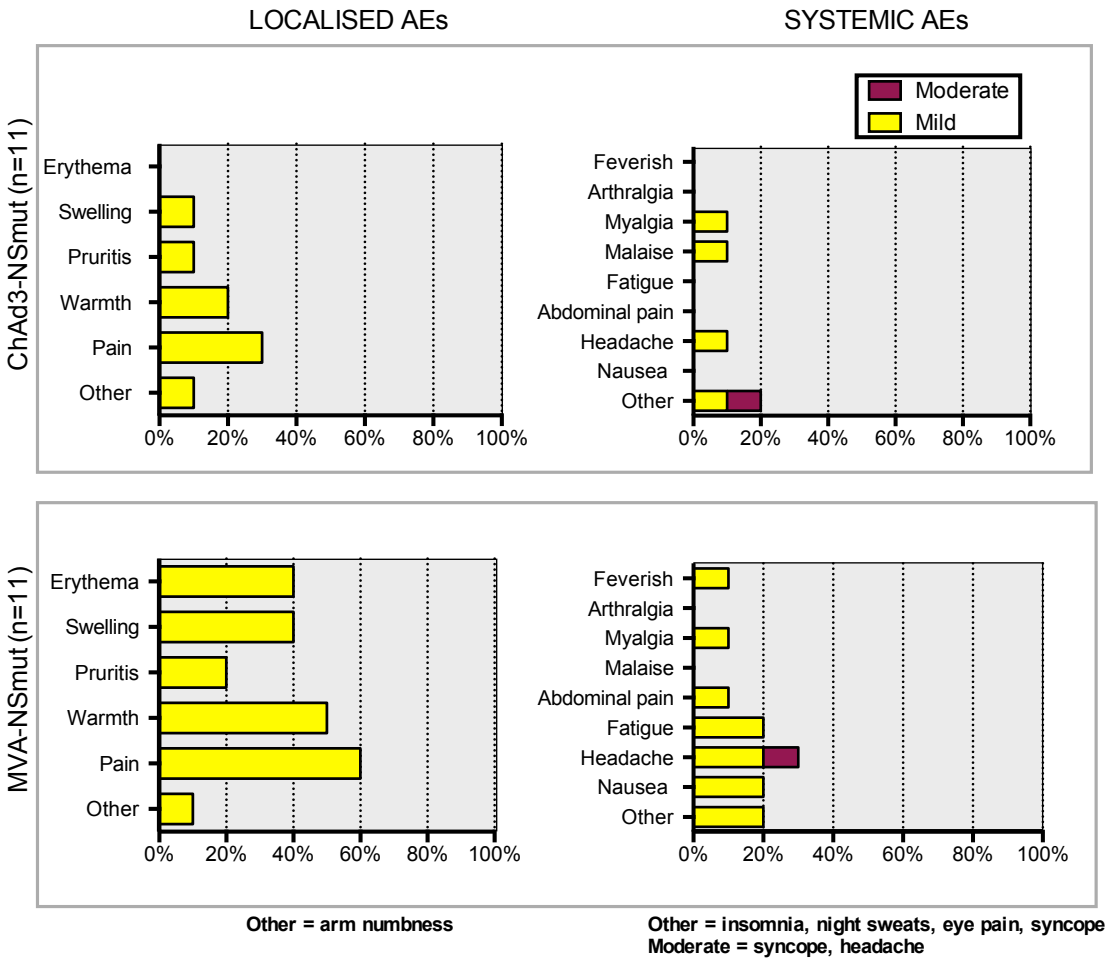
### 3.5.1 ChAd3-NSmut PRIME VACCINATION

All AEs related to ChAd3-NSmut vaccination were of mild severity. The most common local AE was pain (30% of vaccinees), lasting a median of 5 days. Systemic ChAd3-NSmut AEs were infrequent with myalgia, malaise and headache occurring in 9% of those vaccinated.

### 3.5.2 MVA-NSmut BOOST VACCINATION

Local AEs were more common following boosting with MVA-NSmut than ChAd3-NSmut priming. The most common local AEs were pain (60% of subjects, median duration 3.4 days) at the vaccination site, and warmth (50%, median duration 2.8 days). Two systemic AEs were of moderate severity (syncope and headache) in separate patients, while the remaining AEs were all mild. These included; self-limiting headache (30%, median 3.5 days), nausea (20%), fatigue (20%), myalgia (10%) and abdominal pain (10%).

Figure 3-2; Adverse events following vaccination (with definite, probable or possible causal relationships)



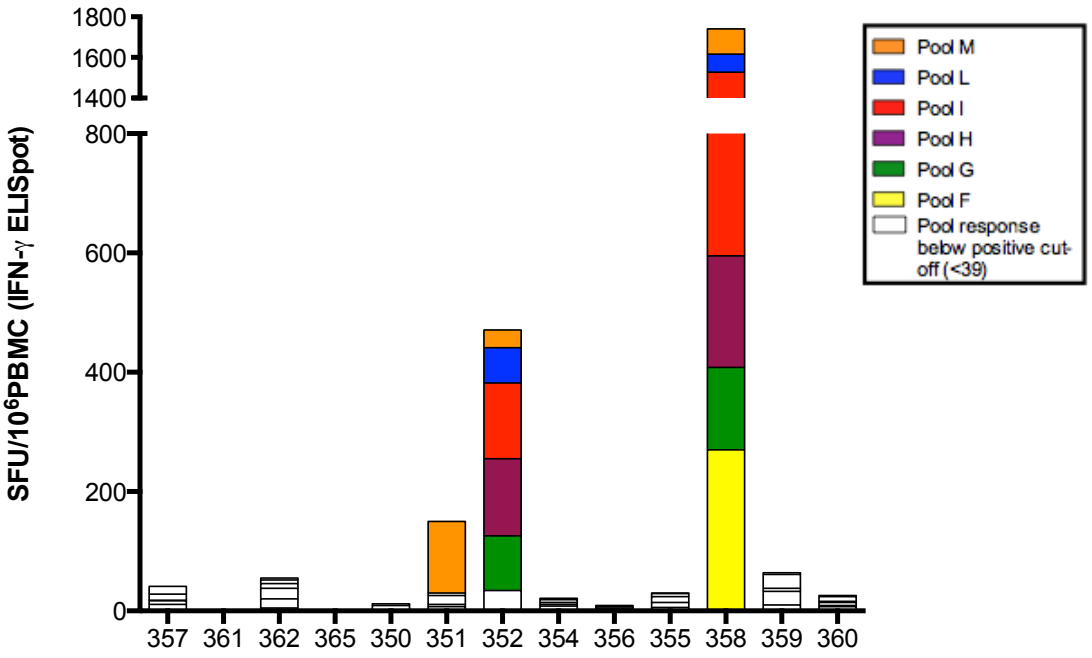

Vaccine was administered intramuscularly. AE= Adverse Event. Left column = local AEs. Right column = systemic AEs. Top panel presents data relating to ChAd3-NSmut vaccination. Bottom panel presents data relating to MVA-NSmut boost vaccination.

## 3.6 KEY QUESTION 2 - Prior to vaccination, can T cells that recognise HCV non-structural peptides be detected in HCV infected trial participants, and if so, what do they target?

### 3.6.1 BASELINE IFN-γ ELISPOT RESPONSES TO HCV NS PEPTIDES INCLUDED IN VACCINE IMMUNOGEN

Prior to vaccination, 3 of the 13 HCV infected patients enrolled in the HCV003 study had positive T cell IFN-γ ELISpot responses to HCV non-structural peptides that correspond to the vaccine insert (Figure 3-3).

Figure 3-3; Summed IFN-γ ELISpot responses to the NS region from individual patients at baseline




Responses are summed responses to pools containing NS proteins in the vaccine immunogen. Only positive pools (defined as ≥39 SFU/$10^6$ PBMCs – see Methods 2.6.2) are shaded in colour. Those pools with responses that fell below the positive cut-off in individual patients are left unshaded.

When possible, positive pool responses were fine mapped to identify the individual 15mer peptide targets of responding T cells. Using this approach 5 peptides (15 amino acids in

length) to which patients had pre-existing T cell responses were identified - 4 of these were identified in patient 358 and the 5th in patient 351 (Table 3-2). No mapping could be performed for patient 352, the 3rd patient with detectable responses at baseline, due to insufficient cell availability.

Table 3-2; Peptides to which T cell IFN-γ ELISPOT responses were detectable prior to vaccination

| 15mer peptide | Nucleotide Location | Responding patient | Epitope* | Predicted HLA-restriction |
|---|---|---|---|---|
| AYAAQGYKVLVLNPS | 4068-4112 | 358 | AYAAQGYKVL^ | Cw03^ |
| PASSQLDLSGWFVAG | 9219-9263 | 351 | QLDLSGWFV* | A2 |
| LTTGSVVIVGRIILS | 5364-5308 | 358 | TGSVVIVGR* | A68 |
| TEVDGVRLHRYAPAC<br>PEFFTEVDGVRLHRY<br>(overlapping peptides)<br>→ TEVDGVRLHRY | 6708-6740 | 358 | EVDGVRLHRY* | A3/B44 |
| LEFWESVFTGLTHID | 5014-5048 | 358 | LEFWESVFT* | B60 |

^previously reported epitope.(Hakamada et al. 2004) *most likely epitope - as predicted by epitope prediction program (either the 9 or 10mer epitope with the highest prediction score and an appropriate HLA type based on responding patient HLA-alleles is given)

Of the 15mer peptides identified by fine mapping, only 1 of 5 contained an immunogenic epitope (AYAAQGYKVL) that has previously been reported in the setting of natural HCV infection (Hakamada et al. 2004). However, this epitope is reportedly restricted to HLA-Cw*03, an allele that was not present in our responder (Patient 358). Another peptide (PASSQLDLSGWFVAG) contained an HLA-A2 restricted epitope (SQLDLSGWF) that was recently described in a paper investigating rational vaccine design by epitope prediction (Guo et al. 2012). However, in-vitro testing of this predicted optimal by IFN-γ ELISpot in the HCV003 patients who had responded to the corresponding 15mer did not elicit T cell responses. The epitope prediction program (http://www.syfpeithi.de) was next employed to predict the optimal peptide for this, and the 4 other identified immunogenic peptides (2 overlapping) (Table 3-2). These predicted optimal peptides were not tested in-vitro in the current study.

### 3.6.2 WHAT IS THE AUTOLOGOUS VIRAL SEQUENCE IN REGIONS CORRESPONDING TO T CELL EPITOPES DETECTED BY IFN-γ ELISpot AT BASELINE?

At baseline, circulating T cells targeting HCV could be detected and mapped to 5 non-structural peptides in patients 351 and 358. The autologous virus was next sequenced at the 5 regions corresponding to these peptides (Table 3-3). For comparison, circulating viral sequences in patients who had negative IFN-γ ELISpot responses to these epitopes were also evaluated.

Table 3-3; IFN-γ ELISpot T cell responses at baseline. Circulating viral sequence vs. vaccine insert at immunogenic epitopes

| Pt. | Arm | QLDLSGWFV | EVDGVRLHRY | TGSVVIVGR | AYAAQGYKVL | LEFWESVFT |
|---|---|---|---|---|---|---|
| 357 | B1 | ---------T | -L--------F | --C------- | ---------- | ----G----- |
| 361 | B1 | ---------- | n.d | n.d | n.d | n.d |
| 362 | B1 | ------N--T | -L--------F | --C------V | ---------- | ----G----- |
| 364 | B1 | ---------T | -L-------TF | n.d | n.d | n.d |
| 365 | B1 | R--------- | ---------- | ---------- | n.d | n.d |
| 350 | B2 | ---------T | -L--------F | --C------- | ---------- | ----G----- |
| 351 | B2 | ------S--- | -L--------- | ---------- | n.d | n.d |
| 352 | B2 | ---------- | -L--------F | --C------- | ---------- | ----G----- |
| 354 | B2 | n.d | -L--------F | --C------- | n.d | ----G----- |
| 356 | B2 | ---------- | -L--------- | n.d | n.d | n.d |
| 355 | C | ---------T | -L--------F | --C------- | ---------- | ----G----- |
| 358 | C | R--------T | -L--------- | --C------- | ---------- | ----G----- |
| 359 | C | ---------T | -L--------F | --C------- | ---------- | ----G----- |
| 360 | C | ---------T | -L--------F | --C------- | ---------- | ----G----- |

Circulating viral sequences in individual patients at T cell epitopes identified by IFN- γ ELISpot assay at baseline. Peptide sequences at the top of each column represent the optimal epitope predicted within the respective vaccine peptide. In columns below, dashes indicate identical amino acid to the vaccine peptide in individuals sequenced. Shaded boxes indicate patients who had detectable T cell responses at baseline to the relevant epitope. n.d = not done.

The autologous viral sequence differed by at least 1 amino acid in 4 of the 5 antigenic vaccine epitopes in responding individuals. It is possible these represent escape variants that are not recognised by circulating T cells. The viral sequence at the 5th epitope (AYAAQGYKLVNPS) identified in patient 358 matched the vaccine peptide. A separate study that will examine pre-vaccine T cell responses in patient 358 in further detail (to

assess recognition of predicted optimal epitopes and cross-recognition of variants) is planned.

## 3.7 KEY QUESTION 3 - Does ChAd3-NSmut and MVA-NSmut vaccination induce T cells in HCV infected patients?

A positive response to vaccination was defined by IFN-γ ELISpot as either of:

i) Any new response of ≥ 39 SFU/$10^6$ PBMCs to an individual peptide pool containing vaccine immunogen (pools F - M) that was not present at baseline,

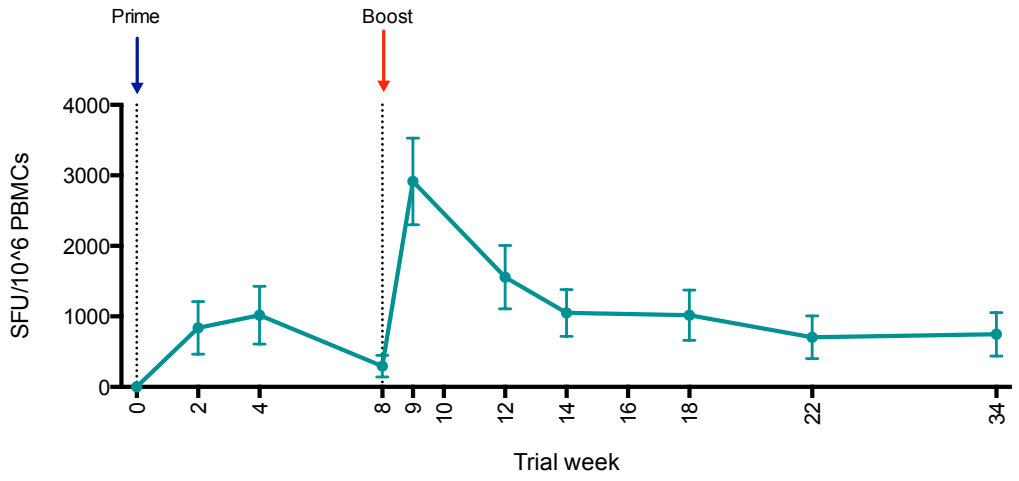
**OR**

ii) An increase in a peptide pool response that was positive prior to vaccination of > 30% (see Methods 2.6.2, page 76)

### 3.7.1 CAN T CELLS BE INDUCED BY VACCINATION IN HEALTHY VOLUNTEERS?

The capacity of ChAd3-NSmut/MVA-NSmut vaccination to induce T cell responses was first evaluated in 10 healthy volunteers (Arm A of the HCV003 study). Although I was involved in trial protocol design for this arm, I did not manage these volunteers, or perform the immunological assays used to assess their vaccine responses. Therefore, only a brief summary of their results is presented here – this is essential because it provides important comparative data for HCV infected patients who received vaccination, and is not yet published (Figure 3-4).

Figure 3-4; Magnitude and kinetics of T cell responses after vaccination with ChAd3-NSmut (prime) and MVA-NSmut (boost) in healthy volunteers (Arm A2)



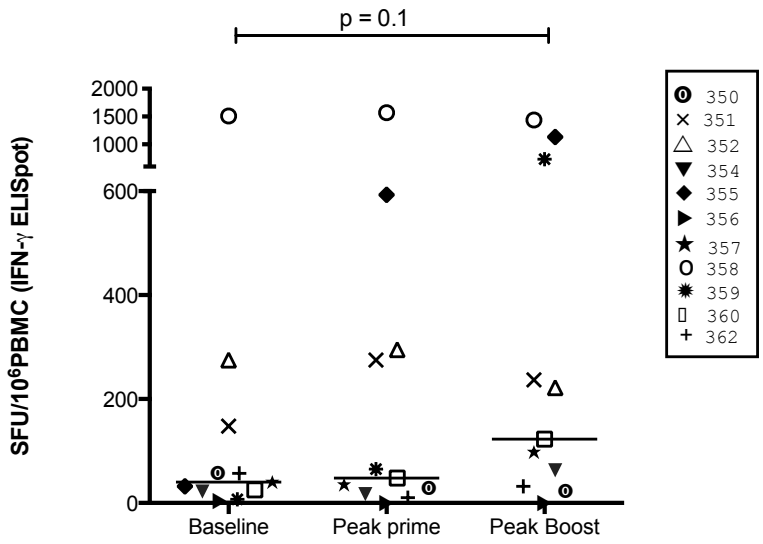

The y-axis is mean (±SEM) summed ex vivo IFN-γ ELISpot responses to NS peptides in the vaccine immunogen from 10 healthy volunteers enrolled in Arm A2 of the HCV003 trial. Vertical dotted lines indicate timing of prime/boost vaccination relative to study commencement. As expected, Arm A1 patients who received MVA vaccine alone did not mount T cell responses to vaccine immunogen.

All 10 of the healthy volunteers in Arm A developed novel anti-HCV T cell responses after vaccination with a median peak summed IFN-γ ELISpot response to vaccine peptides of 807 SFU/$10^6$ PBMCs following prime vaccination, and 2193 SFU/$10^6$ PBMCs after boosting. Importantly, longevity of these responses is demonstrated by the fact that T cells targeting vaccine immunogen are still detectable 26 weeks after boosting in all 9 responders.

### 3.7.2 CAN T CELLS BE INDUCED BY VACCINATION IN HCV INFECTED PATIENTS?

Peak summed IFN-γ ELISpot responses at baseline, and following prime (AdCh3-NSmut) and boost (MVA-NSmut) vaccination for the 11 patients enrolled in HCV003 trial are demonstrated in Figure 3-5. There was no significant difference in T cell responses across time for the whole group based on this analysis (p = 0.1).

Figure 3-5; Magnitude of IFN-γ ELISpot responses to NS HCV peptide pools in all patients (Arms B &C) vaccinated with ChAd3-NSmut and MVA-NSmut



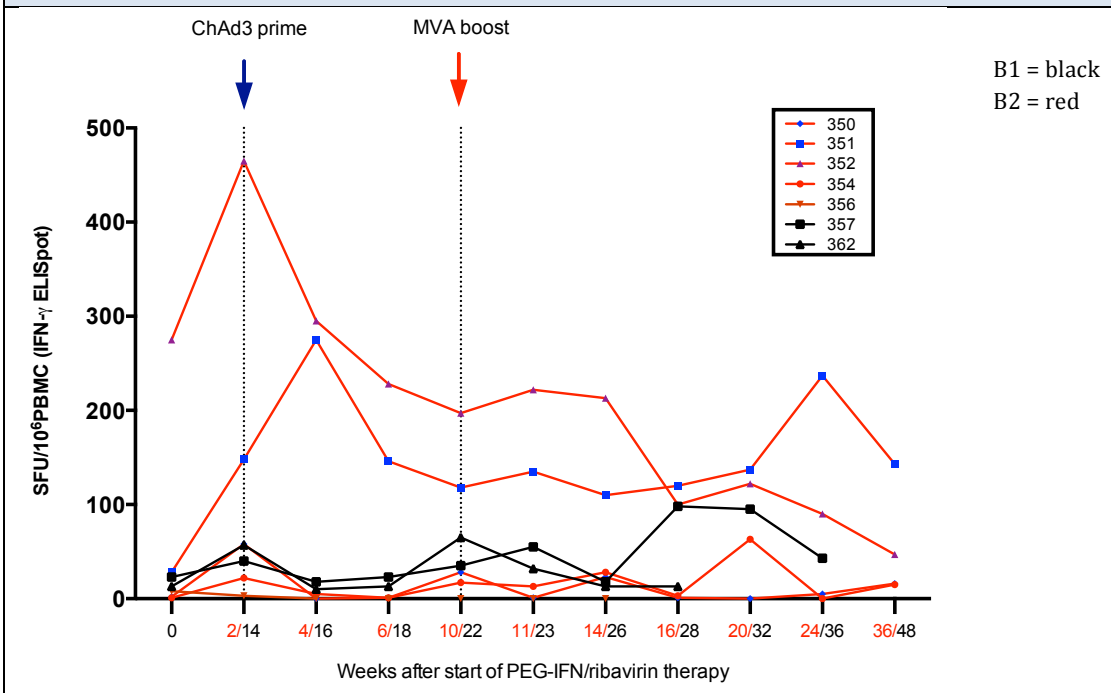

Prime vaccine = ChAd3-NSmut. Boost vaccine = MVA-NSmut. Responses represent summed responses to the NS proteins in the vaccine immunogen. Horizontal lines represent median responses. Values grouped in columns according to peak response at baseline, prime and boost. T cell responses represent summed responses to peptide pools containing the vaccine peptides only (pools F-M).

Evaluating IFN-γ ELISpot responses to individual peptide pools, demonstrated that 5 of the 11 vaccinated patients had developed novel T cell responses after vaccination – 2 of these were in Arm B, and 3 in Arm C. Detailed examination of these responses and their kinetics will be presented according to the respective trial arm in the following sections.

### *Arm B - T cell responses in patients who received vaccination during concurrent PEG IFN/ribavirin therapy*

The total magnitude of the HCV specific T cell response was assessed over time. Timing of prime/boost vaccination relative to therapy did not demonstrate any clear effect on vaccine responses (late prime; Arm B1 after 14 weeks therapy, or early prime; Arm B2 after 2 weeks therapy) (Figure 3-6). It was not evident from summed IFN-γ ELISpot responses to antigenic pools that any individual had responded to vaccination.

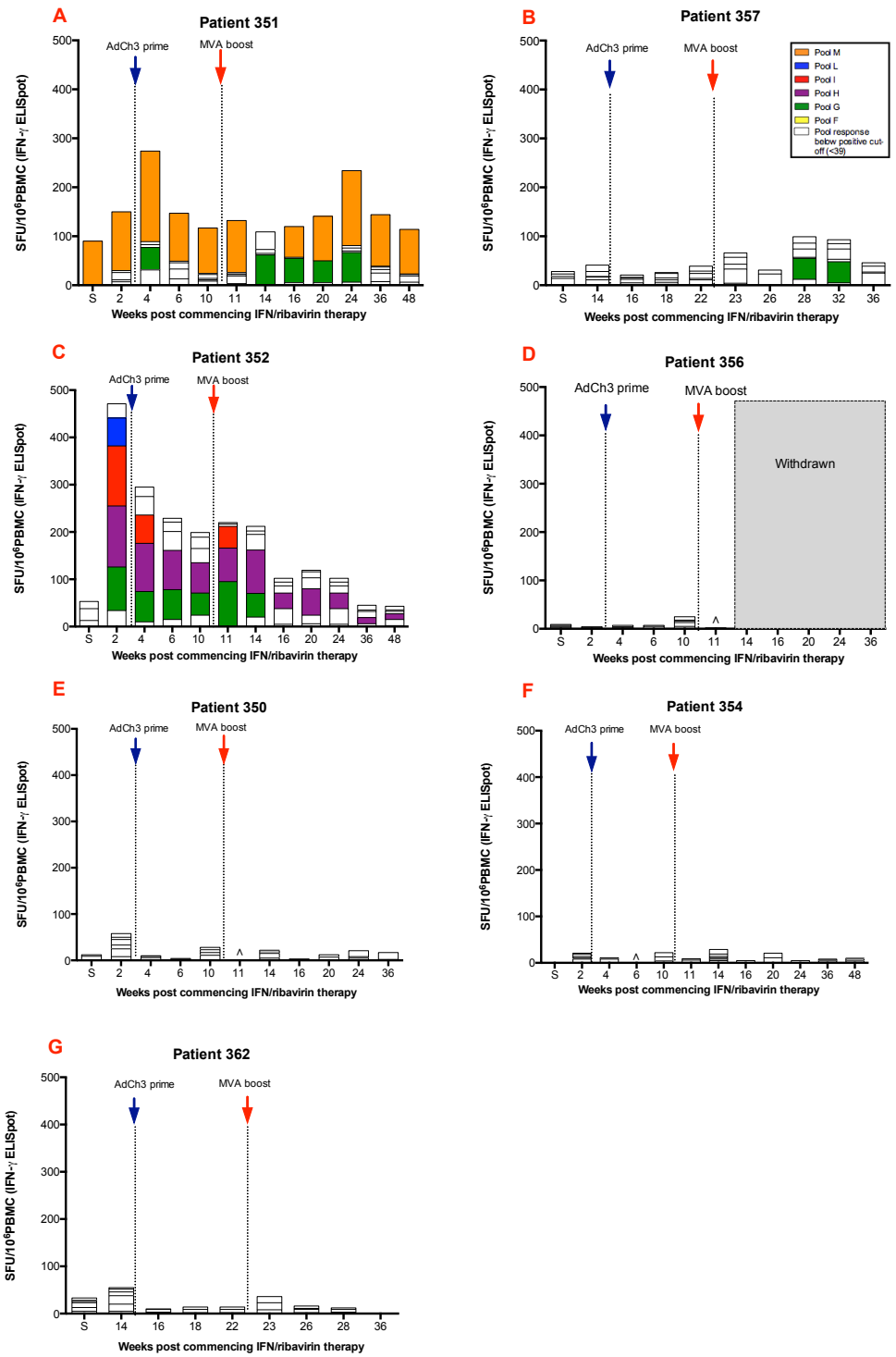
Figure 3-6; The magnitude and kinetics of T cell responses after vaccination with ChAd3-NSmut (prime) and MVA-NSmut (boost)




Kinetics of ex-vivo IFN-γ ELISpot responses over time for individual HCV003 patients. These patients (Arm B) received prime vaccination either after 2 weeks therapy (red lines) or 14 weeks (black lines) PEG-IFN/ribavirin therapy, followed 8 weeks later by boost vaccination. T cell responses are summed responses to peptide pools containing the NS proteins in the vaccine insert. Dotted vertical lines indicate timing of prime and boost vaccination.

However, when individual antigenic pool responses were assessed, it became clear that 2 of the 7 individuals in this trial arm had responded to vaccination (Figure 3-7-A&B). Patient 351 developed novel Pool F & G responses after prime vaccination, while Patient 357 developed a novel Pool G response after boost vaccination.

Figure 3-7; IFN-γ ELISspot responses to NS HCV peptide pools over time in individual patients during PEG-IFN/ribavirin therapy

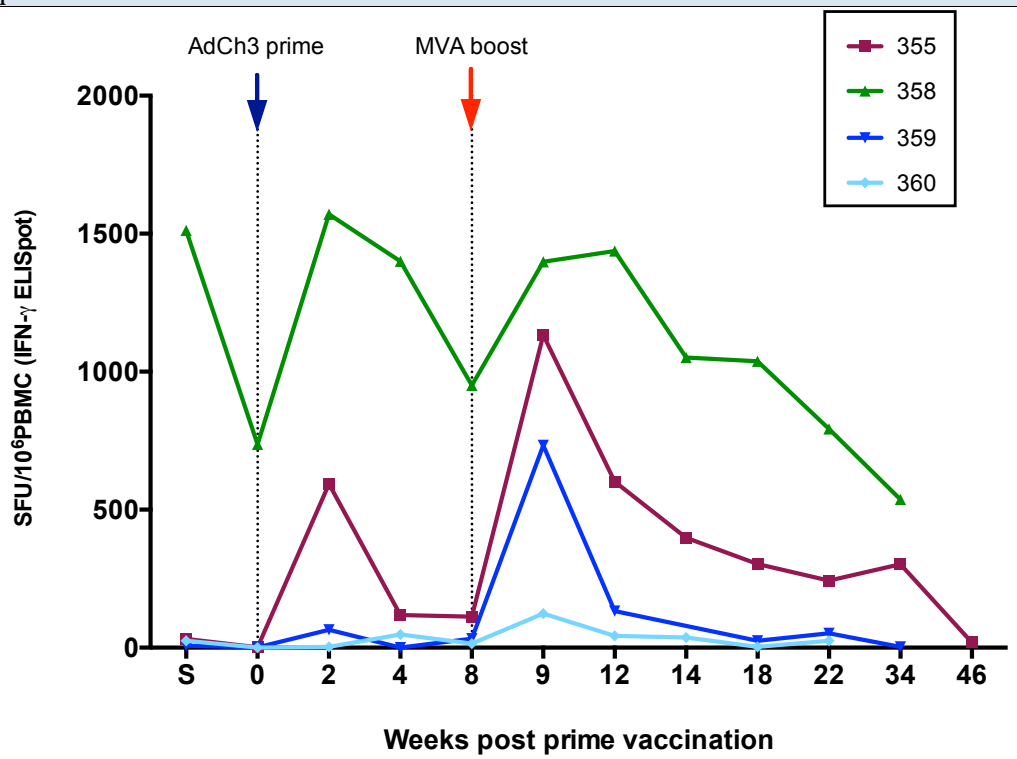



Time of prime/boost vaccination is indicated by vertical dotted lined. Each bar represents the summed IFN-γ ELISpot response at an individual time-point to NS peptides. Colours within each bar indicate the contributing response of each peptide pool (see legend). Only if a pool is positive (≥39 SFU) at any time-point is that subsequently shaded for all time-points – otherwise it is left unshaded. ⋀ = assay performed with a negative response

### *Arm C - T cell responses in HCV infected patients who received vaccination alone*

The total magnitude of the HCV specific T cell response was assessed over time (Figure 3-8). Prime vaccination with ChAd3-NSmut induced novel T cell responses in 1 patient (Pt. 355, peak 2 weeks post prime = 593 SFU/$10^6$ PBMCs). Boosting with MVA-NSmut induced T cell responses in another 2 patients (Pt. 359 and Pt. 360).

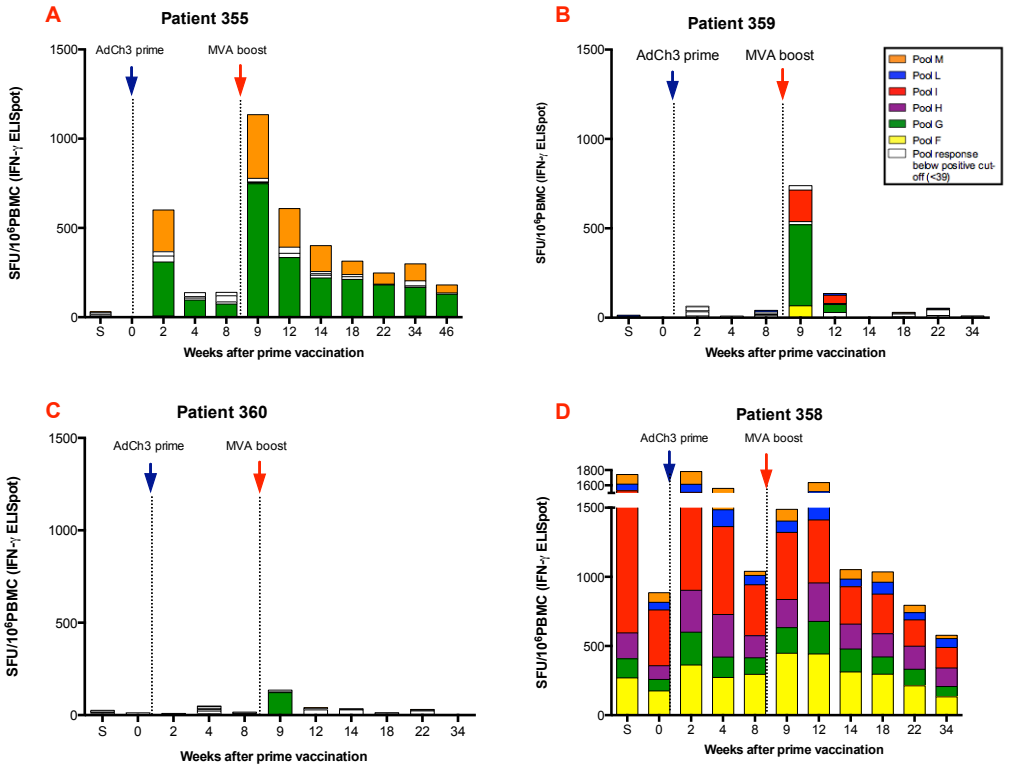
Figure 3-8; Magnitude and kinetics of T cell responses after prime/boost vaccination for patients enrolled in Arm C




Prime vaccine = ChAd3-NSmut. Boost vaccine = MVA-NSmut. Kinetics of ex vivo IFN-γ ELISpot responses over time by individual. These patients (Arm C) received vaccination alone without concurrent anti-HCV therapy. Coloured lines connect individual patient responses over time. T cell responses represent summed responses to peptide pools containing the vaccine peptides. S = screening.

The kinetics of individual peptide pool responses is demonstrated in Figure 3-9. All 3 vaccine-induced T cell responses peaked 1 week after boost vaccination. Patient 358 had high pre-existing T cell responses with no evidence that vaccination boosted these responses or induced new ones.

Figure 3-9; IFN-γ ELISspot responses to NS HCV peptide pools over time in individual patients in Arm C

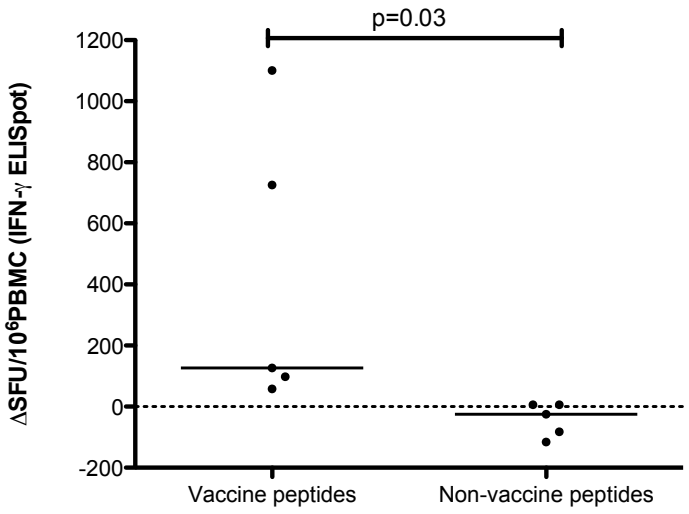



Time of prime/boost vaccination is indicated by vertical dotted lined. Each bar represents the summed IFN-γ ELISpot response at an individual time-point to NS peptides. Colours within each bar indicate the contributing response of each peptide pool (see legend). Only if a pool is positive (> 41 SFU) at any time-point is that subsequently shaded for all time-points – otherwise it is unshaded. S = screen

### 3.7.3 VACCINE RESPONDERS – IS T CELL INDUCTION LIMITED TO PEPTIDES WITHIN THE VACCINE?

If novel T cell responses are directly induced by vaccination, they should be limited to HCV peptides included within the vaccine insert (and possibly the vaccine vector). To confirm this, IFN-γ ELISpot assays using peptide pools corresponding to HCV antigen (core and NS2) not included in the vaccine were also assessed – as expected these responses did not change from baseline in vaccine responders (p = 0.03, Wilcoxon matched pairs, one-tailed) (Figure 3-10). In addition, no vaccine recipient developed a novel T cell response to any HCV antigen pool not included in the vaccine across the study period (see Appendix 6 and Appendix 7).

Figure 3-10; Peak change in IFN-γ ELISpot from baseline following boost vaccination in vaccine responders: vaccine peptides vs. non-vaccine peptides




IFN-γ ELISpot responses to vaccine peptides and IFN-γ ELISpot responses to HCV peptides not included in the vaccine insert (core and NS2). The p value is calculated using Wilcoxon matched pairs analysis for non-parametric data. Change is calculated by subtracting the peak pre-vaccine response from the peak boost response.

T cell immunity against the adenovirus vector was also assessed longitudinally by IFN-γ ELISpot assay (Appendix 8 and Appendix 9). The peptide pool employed for this assay was based on the hexon protein from human adenovirus serotype 5 (not ChAd3) because this peptide pool is commercially available (Miltenyi ♯130-093-495), and there is significant

homology between these subtypes in their hexon proteins. Of the HCV infected vaccine recipients, 9/11 had detectable T cell immunity to adenovirus hexon before vaccination. All these responses increased after priming (ChAd3-NSmut). There was no suggestion that successful induction of T cell responses against vaccine immunogen related to T cell immunity against hexon (either at baseline or after priming).

### 3.7.4 MAPPING OF T CELL RESPONSES IN HCV INFECTED PATIENTS

#### *WHAT IS THE BREADTH OF VACCINE INDUCED T CELL RESPONSES?*

Of the 5 HCV infected vaccine responders, patient 359 had the greatest breadth of response to vaccination with new responses detectable in 3 separate peptide pools (Figure 3-9-B) whilst 1 patient (Pt. 355) responded to 2 pools, and the remaining 3 patients (Pts. 351, 357 and 360) developed novel responses to just 1 pool. This is in contrast to healthy volunteers enrolled in Arm A of the HCV003 study where 3 volunteers developed T cell responses to all 6 peptide pools, and the average number of pools to which vaccine induced responses were detected was 4 (unpublished data).

#### *WHAT PEPTIDES DO VACCINE INDUCED T CELLS TARGET?*

Using the fine mapping technique described in Section 2.6.3 (page 77), 6 of the 8 vaccine induced IFN-γ ELISpot pool responses could be mapped to 4 individual peptides (Table 3-4). Two of these were overlapping peptides identified in 1 patient. The remaining pools could not be fine mapped, either due to insufficient cell number at relevant time-points, or IFN-γ ELISpot responses that were too weak. All identified peptides contained previously reported, immunogenic epitopes in the setting of natural HCV infection (Cerny et al. 1995; Wang & Eckels 1999; Guo et al. 2012).

Table 3-4; Fine mapping of positive peptide pools identified 3 optimal epitopes induced by vaccination

| 15mer peptide | Optimal epitope | Location | HLA restriction | Responding patients |
|---|---|---|---|---|
| LAAKLSGLGINAVAY | KLSGLGINAV[1] | $NS3_{1406}$ | HLA-A2 | 351, 355, 360 & 357 |
| LIFCHSKKKCDELAA | HSKKKCDEL[2] | $NS3_{1395}$ | HLA-B8 | 359 |
| IYHSLSRARPRWFML/ LSRARPRWFMLCLLL (overlapping) | SRARPRWFM[3] | $NS5B_{2984}$ | HLA-A2 | 355 |

All 3 of these peptides contained previously reported immunogenic epitopes in HCV infection.
[1] Cerny et al. 1995 [2] H. Wang & Eckels 1999 [3] Guo et al. 2012

Epitope $NS3_{1406}$ was identified as most immunogenic with 4 of the 5 HLA-A2 positive patients developing responses that targeted this epitope.

As an example of the difficulties with fine mapping, despite also developing IFN-γ ELISpot responses to pools F, L and M, only the pool G response in patient 359 could be mapped to $NS3_{1406}$ (Figure 3-9-B). This was due to insufficient cell availability at week 9, the only time-point at which significant, but transient IFN-γ ELISpot responses were detectable.

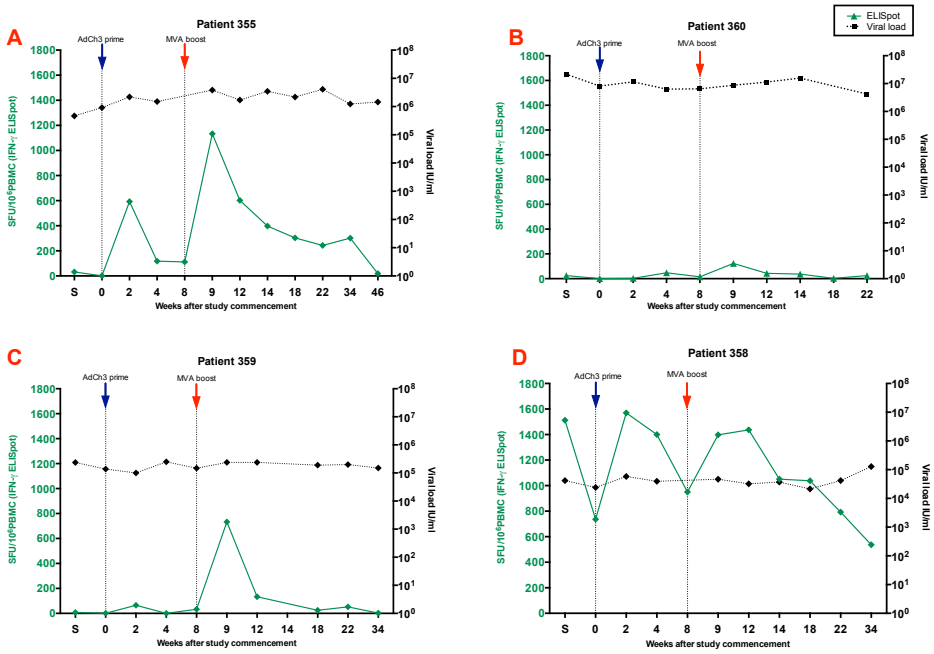
## 3.8 KEY QUESTION 4 – Do vaccine induced T cells effectively target circulating virus?

Theoretically, if vaccine induced T cells effectively target circulating virus, a decrease in viral load might be expected. For this reason, viral load and its relationship to T cell kinetics was assessed.

### 3.8.1 IS THERE A RELATIONSHIP BETWEEN VACCINE INDUCED IFN-γ ELISPOT RESPONSES AND HCV VIRAL LOAD?

The relationship between circulating viral load and vaccine induced T cell responses is best evaluated in Arm C patients because they do not have the confounding effect of PEG-IFN/ribavirin therapy on viral load. Despite successful induction of novel anti-HCV T cells by vaccination in 3 of the 4 patients in this group, there was no significant change in viral load for any patient (Figure 3-11).

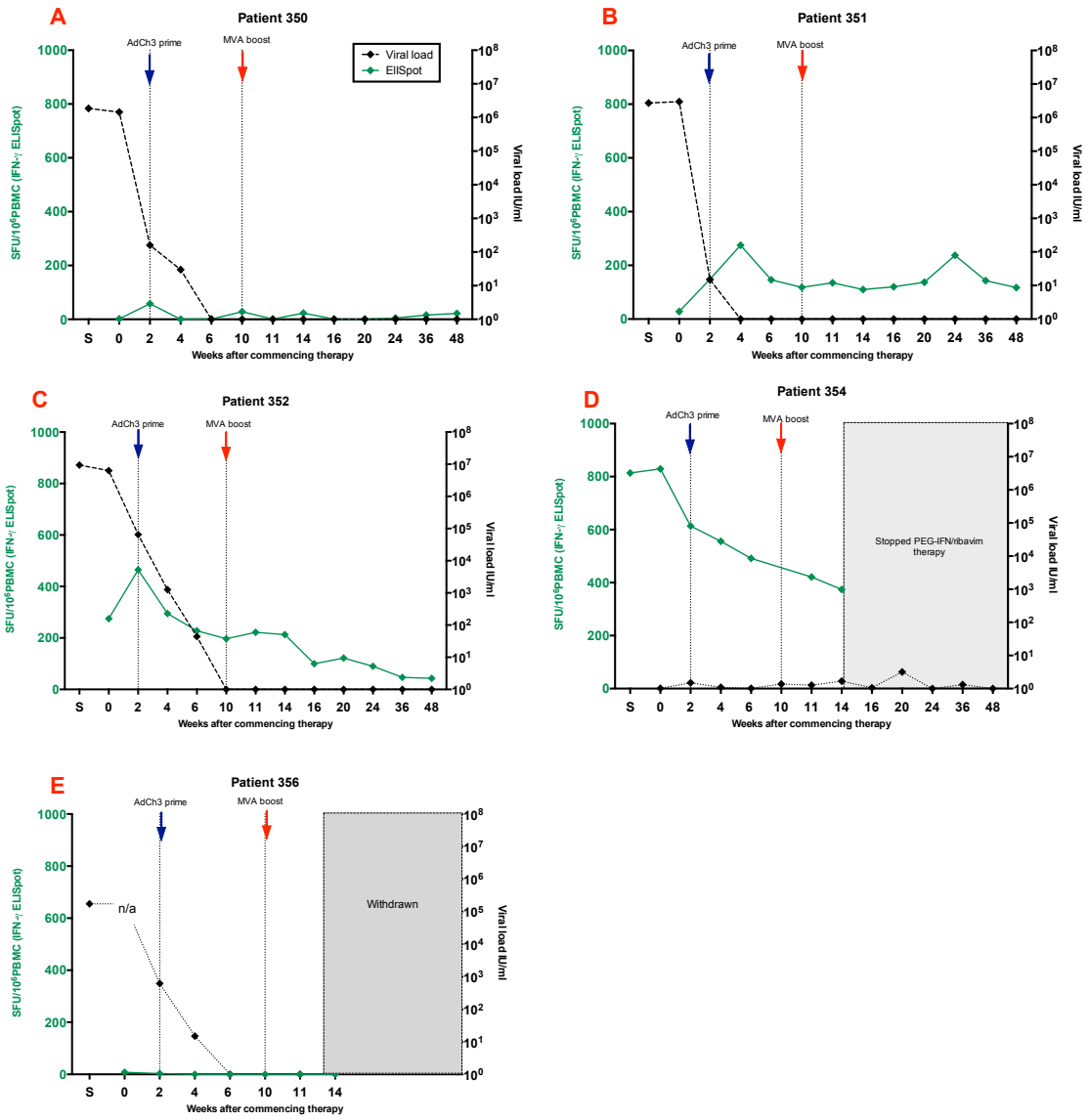
Figure 3-11; Longitudinal change in viral load relative to IFN-γ ELISpot response in individual patients in Arm C




Right Y-axis = viral load, (black dotted line). Left Y-axis = summed IFN-γ ELISpot response to vaccine immunogen pools (green line). Vertical dotted lines indicate timing of prime/boost vaccination. Patients 355 (A), 360 (B) and 359 (C) were vaccine responders.

For patients in Arm B, longitudinal decreases in viral load are consistent with the known effect of PEG-IFN/ribavirin therapy. Example plots for patients enrolled in the early vaccination arm (B2) during therapy are presented in **Figure 3-12**.

Figure 3-12; Longitudinal change in viral load relative to IFN-γ ELISpot response in individual patients in Arm B2




Right Y-axis = viral load, (black dotted line). Left Y-axis = summed IFN-γ ELISpot response to vaccine immunogen pools (green line). Vertical dotted lines indicate timing of prime/boost vaccination. Patients 355 (A), 360 (B) and 359 (C) were vaccine responders.

### 3.8.2 WHAT IS THE SEQUENCE OF CIRCULATING VIRUS AT SITES THAT CORRESPOND TO VACCINE INDUCED T CELL EPITOPES?

One possible reason that T cell induction by vaccine did not cause a decrease viral load for patients in Arm C is that vaccine immunogen differs significantly from the corresponding circulating HCV sequence. If this is the case, T cells induced by vaccination may not recognise autologous virus. To explore this possibility, the autologous virus was sequenced at viral regions that correspond to the T cell antigenic targets in all trial participants at baseline. In addition, when possible, virus was sequenced after vaccination to determine whether T cell induction was associated with a change in viral sequence. A summary of these results is presented in Table 3-5.

Table 3-5; Vaccine induced responses - Circulating viral sequence vs. vaccine insert

| Pt. | Geno type | Arm | | $NS3_{1406}$ epitope | Freq. (%) | $NS5B_{2984}$ epitope | Freq. (%) | $NS3_{1395}$ epitope | Freq. (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | KLSGLGINAV | 1a: 0.0<br>1b: 4.1 | SRARPRWFM | 1a: 0.2<br>1b: 1.8 | HSKKKCDEL | 1a: 87.2<br>1b: 87.3 |
| 357 | 1a | B1 | | --VA--V--- | 29.3 | --------W | 38.7 | --------- | 87.2 |
| 361 | 1a | B1 | | --VA------ | 44.7 | --------W | 38.7 | --------- | 87.2 |
| 362 | 1a | B1 | | --VA------ | 44.7 | -------LW | 3.2 | --------- | 87.2 |
| 365 | 1b | B1 | | ---S--L--- | 17.1 | P------P- | 0.0 | --------- | 87.3 |
| 350 | 1a | B2 | | --VA------ | 44.7 | --------W | 38.7 | --------- | 87.2 |
| 351 | 1b | B2 | | ---A--L--- | 7.3 | --------- | 79.0 | --------- | 87.3 |
| 352 | 1b | B2 | | --VA--V--- | 0.6 | --------W | 39.1 | --------- | 87.3 |
| 354 | 1a | B2 | | --TAM----- | 0.0 | n.d | | --------- | 87.2 |
| 356 | 1b | B2 | | ------L--- | 37.3 | -------I- | 1.9 | --------- | 87.3 |
| 355 | 1a | C | Pre | --VA--V--- | 29.3 | --------W | 38.7 | --------- | 87.2 |
| | | | Post | --VA--V--- | | --------W | | --------- | |
| 358 | 1a | C | Pre | --VA--V--- | 29.3 | --------W | 38.7 | --------- | 87.2 |
| | | | Post | --VA--V--- | | n.d | | --------- | |
| 359 | 1a | C | Pre | --VA--V--- | 29.3 | --------W | 38.7 | ---R----- | 2.1 |
| | | | Post | --VA--V--- | | n.d | | ---R----- | |
| 360 | 1a | C | Pre | --VA------ | 44.7 | --------W | 38.7 | --------- | 87.2 |
| | | | Post | --VA------ | | n.d | | --------- | |
| Most common 1a | | | | --VA------ | 45.0 | -H------W | 40.7 | --------- | 87.2 |
| Most common 1b | | | | ------L--- | 37.0 | --------- | 79.0 | --------- | 87.3 |

Circulating viral sequences in individual patients at vaccine induced T cell epitopes. Epitope sequences at the top of columns represent the vaccine peptide. Dashes indicate identical amino acid to the vaccine peptide. Shaded boxes indicate individual patients who developed T cell responses following vaccination to the relevant epitope. Pre = viral sequence at baseline. Post = viral sequence 2 weeks following boost vaccination. Where no pre/post is given, this sequence represents viral sequence at baseline. Frequency (freq) relates to the relative frequency of the identified variant amongst ≈ 800 HCV genotype 1a/≈800 genotype 1b sequences reported on the Los Alamos HCV database. n.d = not done.

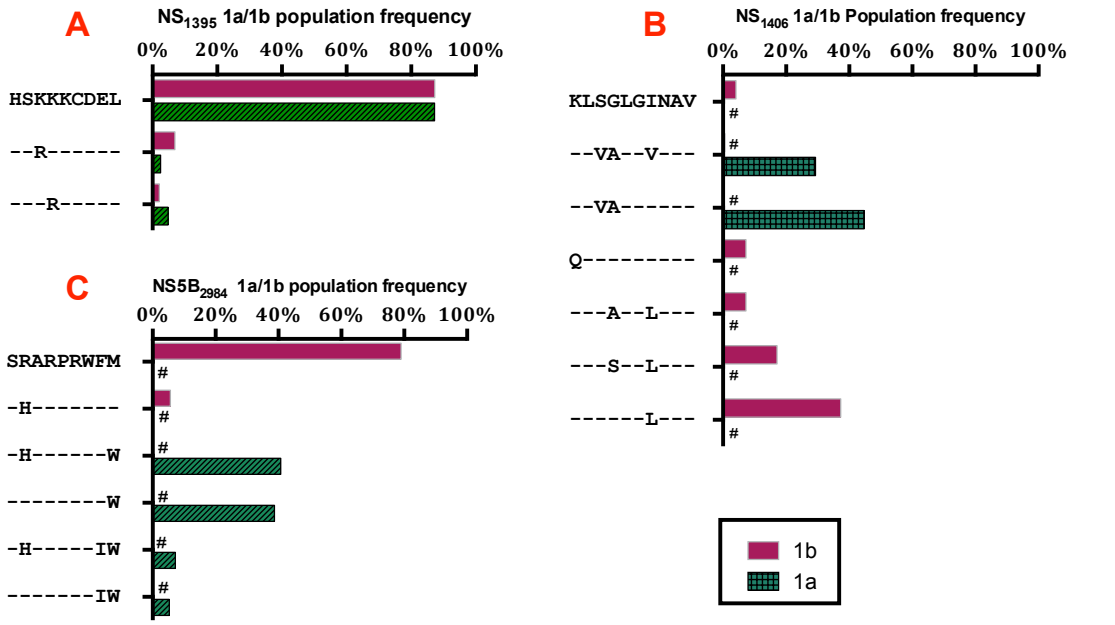
In all instances when vaccination induced a T cell response, the circulating virus at baseline contained a variant sequence at the relevant epitope. In addition, no patient with a viral sequence identical to the vaccine immunogen mounted a T cell response to that epitope following vaccination. Viral sequence was determined 2 weeks after boost vaccination in 3 Arm C patients across 4 vaccine induced T cell epitopes – no change in viral sequence after T cell induction was observed in any of these instances.

### 3.8.3 HOW VARIABLE ARE HCV SEQUENCES AT IMMUNOGENIC EPITOPES TARGETED BY VACCINE INDUCED T CELLS?

The considerable viral sequence diversity identified at T cell epitopes targeted by vaccine induced T cells raised the possibilities that; i) at immunodominant epitopes the vaccine insert might vary significantly from the most common naturally occurring genotype 1 HCV sequences, and therefore ii) T cells induced by vaccination may not recognise circulating virus.

To address the issue of viral sequence variability described above, the relative frequency of the identified immunogenic T cell epitopes and their variants across known genotype 1a and 1b HCV sequences were determined using the Los Alamos HCV database (http://hcv.lanl.gov/content/index) (Figure 3-13). Only confirmed, unrelated sequences were included in this analysis. The number of available sequences for analysis varied for each epitope; for epitope $NS_{1406}$ (1a: n = 839, 1b: n = 809), for epitope $NS5B_{2984}$ (1a: n = 563: 1b: n = 480), and $NS3_{1395}$ (1a: n = 839, 1b: n = 821).

Figure 3-13; Sequence variability at vaccine induced T cell epitopes at a population level in Genotype 1 HCV infection defined using Los Alamos database



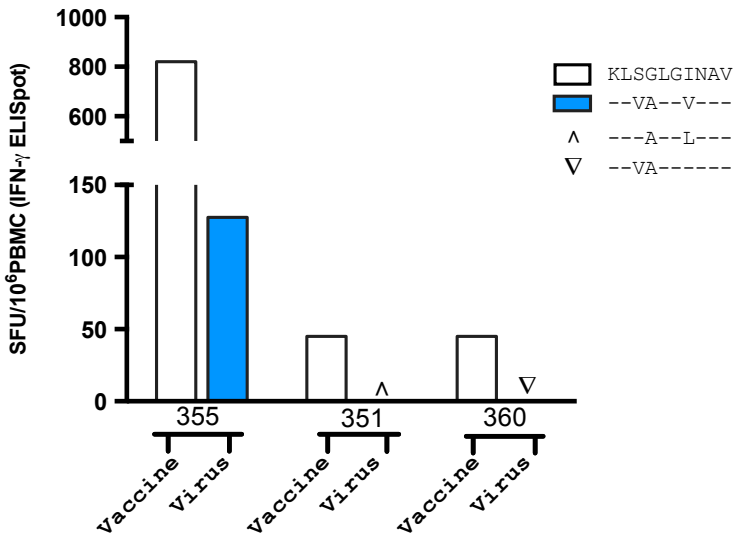

Graph (A) = Epitope $NS3_{1395}$, graph (B) = $NS_{1406}$ and graph (C) = $NS5B_{2984}$. Only epitope variants with a prevalence of >5% (in one of the respective 1a or 1b subpopulations) are presented. Y-axis: the vaccine sequence is given in full – identical amino acids to the vaccine are indicated by a dash. Prevalence amongst genotype 1a sequences (green shading) and genotype 1b sequences (purple shading) are presented. ♯ indicates 0% of sequences for the relevant subtype contained the respective sequence. Frequency is the relative frequency of the identified variant amongst sequences available on the Los Alamos HCV database.

The level of sequence diversity varied significantly across the 3 identified vaccine induced epitopes. $NS3_{1406}$, the most immunogenic epitope was highly variable, with no epitope variant present in more than 50% of all genotype 1a or 40% of genotype 1b sequences. The vaccine sequence at this epitope actually represents a rare variant, present in only 4.1% of genotype 1b, and no genotype 1a sequences. In contrast, $NS3_{1395}$ was highly conserved with the vaccine sequence present in 87.3% of all viral sequences analysed (1a & 1b combined). Interestingly, the 1 patient who developed a vaccine response to $NS3_{1395}$ was also the only trial participant with a variant sequence in circulating virus at that epitope.

### 3.8.4 DO VACCINE INDUCED T CELLS TARGET EPITOPE VARIANTS THAT CORRESPOND TO CIRCULATING VIRUS?

For 3 patients with vaccine induced T cell responses to epitope $NS_{1406}$, T cell cross-recognition of variant peptides matching amino acid sequences of autologous virus was assessed by IFN-γ ELISpot assay (Figure 3-14). Compared with vaccine immunogen $NS_{1406}$, IFN-γ ELISpot responses to variant peptides was substantially reduced, or negative in all 3 patients indicating that T cell cross-recognition is poor.

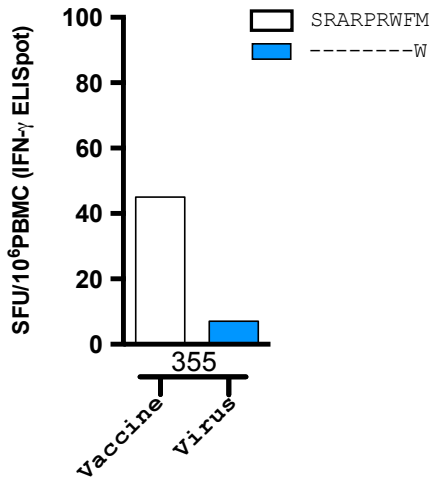
Figure 3-14; T cell responses to peptides corresponding to the vaccine sequence compared with host virus sequence at immunogenic epitopes




Patient numbers are indicated on the x-axis. Columns indicate the IFN-γ ELISpot response (y-axis) to each individual epitope variant. Unshaded columns represent responses to peptide matching vaccine immunogen $NS_{1406}$. Adjacent shaded columns represent variant peptides matching autologous viral amino acid sequence in individual patients (see legend). A response of 0 SFU is indicated by a symbol only (no bar). The IFN-γ ELISpot for patient 355 was performed using fresh PBMCs, while those for 351 and 360 were assessed using frozen PBMCs from the time of peak vaccine induced T cell response.

Similarly, in patient 355, vaccine induced T cells targeting epitope $NS3_{1395}$ poorly cross-recognised the corresponding circulating viral peptide as judged by IFN-γ ELISpot assay (Figure 3-15).

Figure 3-15; IFN-γ ELISpot responses to vaccine immunogen compared with corresponding autologous viral peptide




Response to vaccine epitope $NS_{2986}$ (unshaded column) and viral variant epitope (blue column) in patient 355 using cells from 2 weeks after boost vaccination. The vaccine sequence is indicated in the legend, with the autologous viral sequence below this. Dashes indicated identical amino acids to those of vaccine immunogen.

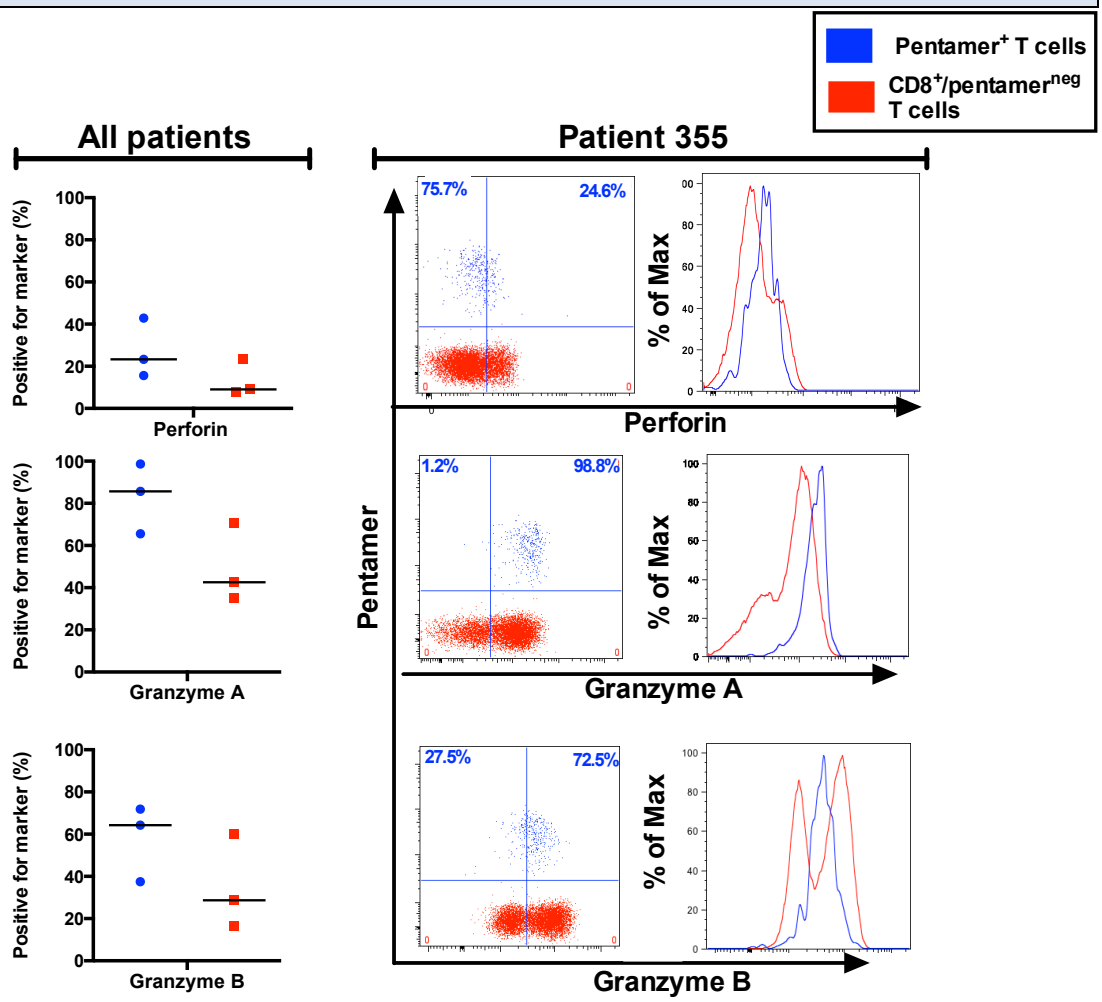
### 3.8.5 WHAT IS THE PHENOTYPE OF VACCINE INDUCED T CELLS?

The cytotoxicity, activation, differentiation and memory phenotype of vaccine induced T-cells was evaluated by flow cytometry using an $NS3_{1406}$ pentamer. Of the 4 patients with vaccine-induced responses to epitope $NS_{1406}$, 3 had sufficient PBMCs stored for analysis using this approach (Pts. 355, 357 and 360). Example FACS plots for patient 355 are presented throughout this section because this patient had the highest number of pentamer reactive cells.

### *CYTOTOXIC CAPACITY*

Compared with the total circulating CD8+ T cell population, vaccine induced T cells are relatively high in perforin, granzyme A and granzyme B (23.3%, 85.7% and 64.3% vs. 9.0% and 42.6% and 28.7% of the pentamer non-reactive CD8+ populations respectively, median values) (Figure 3-16).

Figure 3-16; Granzyme A, Granzyme B and perforin expression on pentamer ($NS3_{1406}$) reactive T cells after boost vaccination

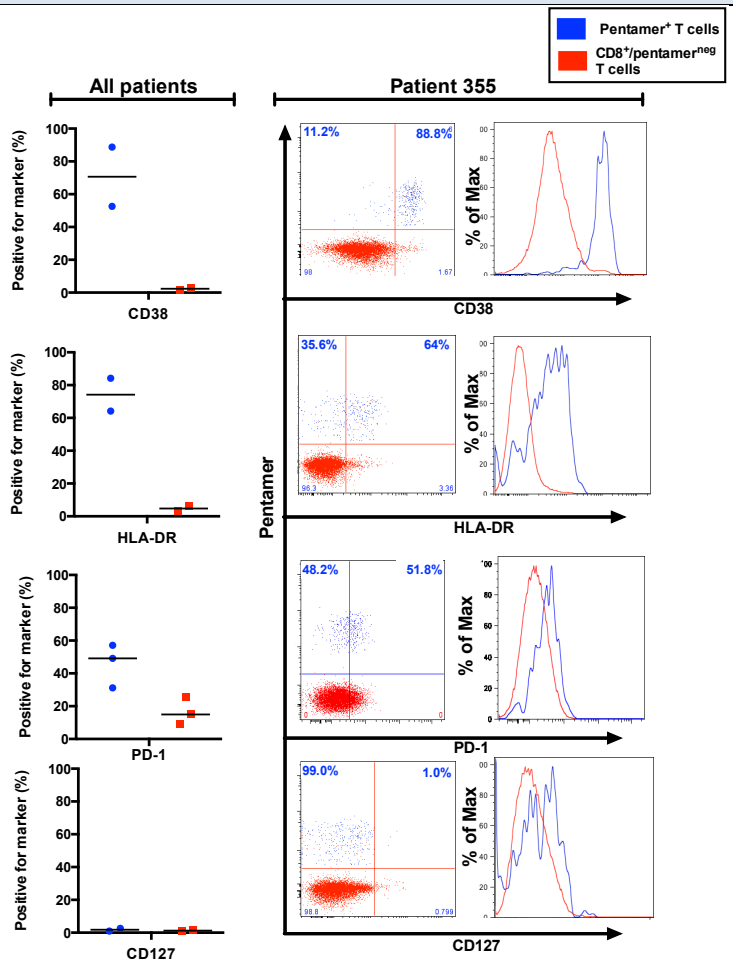



Blue dots/lines represent pentamer reactive $CD8^+$ T cells Red dots/lines represent $CD8^+$ T cells negative for pentamer staining. Left column (All patients): represents collective data from phenotyping of pentamer reactive CD8+ T cells from patients 355, 357 and 360 after boost vaccination. Middle/right columns (Patient 355): example FACS plots and histogram for respective marker. These have first been gated on $CD8^+/CD3^+$ lymphocytes. Max = maximum. Numbers in blue font give the percentage of pentamer cells within the respective gate.

### *ACTIVATION AND DIFFERENTIATION PHENOTYPE*

Compared with total $CD8^+$ T cell populations, vaccine induced T cells are relatively high in CD38, HLA-DR, PD-1 and CD127 (70.6%, 74.2%, 49.2% and 1.8% vs. 2.3%, 4.8%, 15.0% and 1.2% of the $pentamer^-$ $CD8^+$ populations respectively, median values) (Figure 3-17). Expression of CD38, CD127 and HLA-DR by vaccine-induced cells for patient 357 was not assessed due to limited cell availability.

Figure 3-17; CD38, CD127, HLA-DR and PD-1 expression on pentamer ($NS3_{1406}$) reactive T cells after boost vaccination




Blue dots/lines represent pentamer reactive $CD8^+$ T cells Red dots/lines represent CD8+ T cells negative for pentamer staining. Left column (All patients): represents collective data from phenotyping of pentamer non-reactive CD8+ T cells from patients 355, 360 (and 357 for PD-1) after boost vaccination. Middle/right columns (Patient 355): example FACS plots and histogram for respective marker. These have first been gated on $CD8^+/CD3^+$ lymphocytes. Max = maximum. Numbers in blue font give the percentage of cells within the respective gate.

### *MEMORY PHENOTYPE*

The memory phenotype of $CD8^+$ T cells can be defined using CCR7/CD45RA expression as demonstrated in Figure 3-18. Although $CD4^+$ T cells that are $CCR7^-$ $CD45RA^+$ are poorly defined in the literature, throughout this thesis they will be referred to as terminally differentiated effector memory cells.

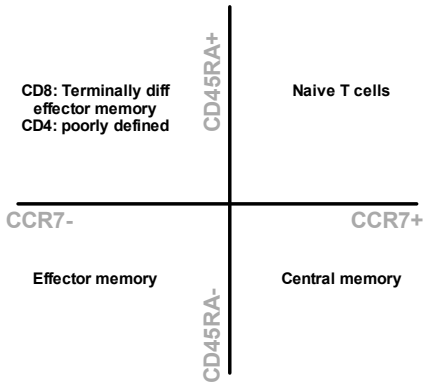
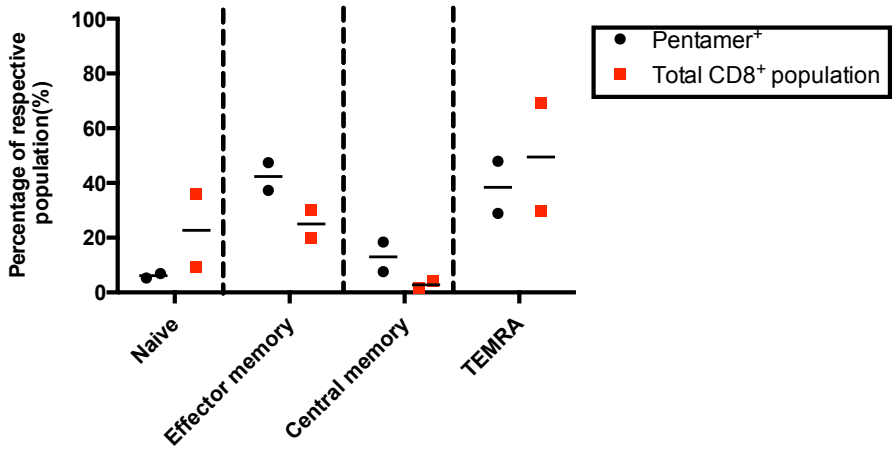



Figure 3-18; Method for defining memory phenotypes; CCR7 and CD45RA expression used in combination as illustrated

Vaccine induced T cells display a variety of memory phenotypes (Figure 3-19). Effector memory cells are most common, followed by terminally differentiated effector memory cells, while naïve cells are least frequently pentamer reactive (42.4%, 38.4% and 6.1% respectively).

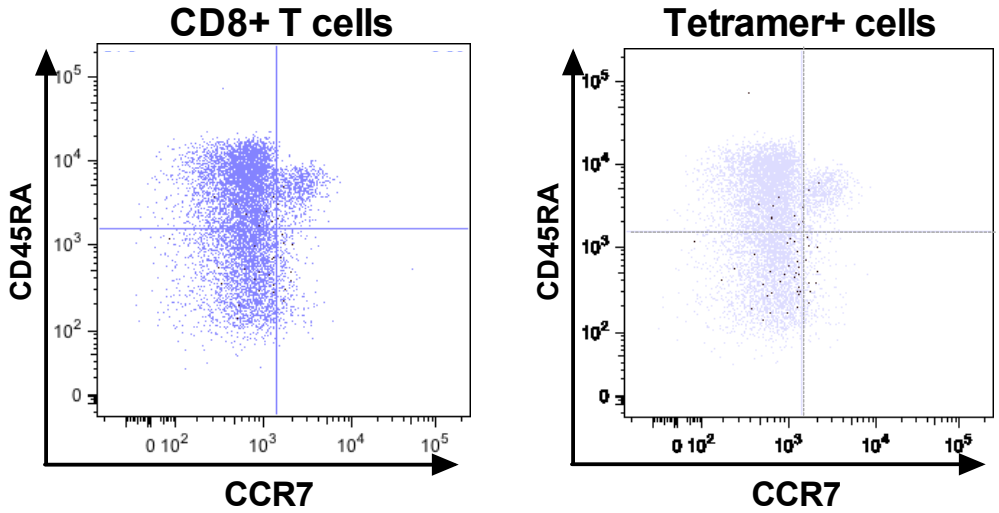
Figure 3-19; Memory phenotype of vaccine induced T cells 1 week after boost vaccination in patients 355 and 360 compared with total CD8+ T cell population




Memory subsets were defined using CCR7 and CD45RA expression (see Figure 3-18). TEMRA = terminally differentiated effector memory cells ($CCR7^-$, $CD45RA^+$)

Example FACS plots of the memory distribution of CD8+ T cells and pentamer reactive T cells for patient 355 are given in Figure 3-20.

Figure 3-20; Memory phenotype of vaccine induced T cells 1 week after boost vaccination in patient 355. CCR7/CD45RA co-expression is demonstrated on this raw FACS plot.

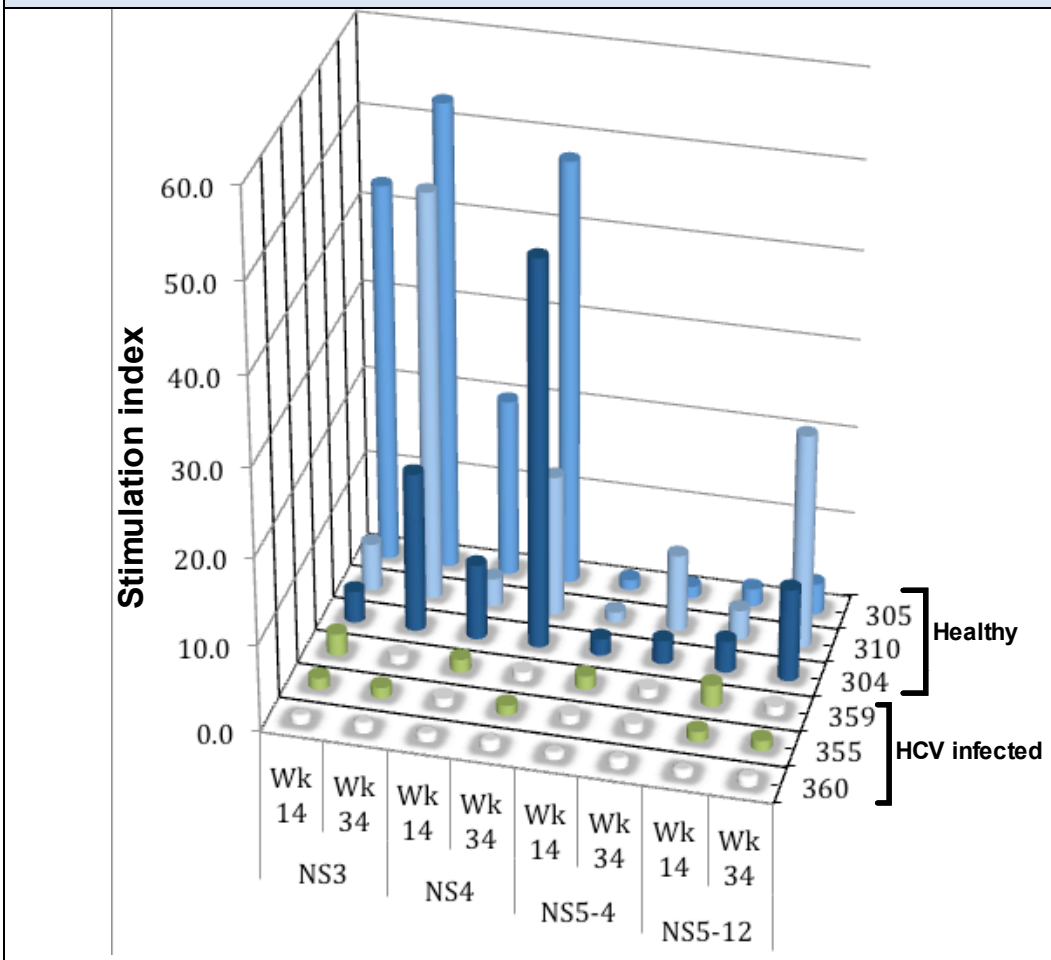



Left graph: The memory phenotype of total $CD8^+$ T cells for comparison with pentamer reactive cells in the right graph. Right graph: pentamer ($NS3_{1406}$) positive T cells are represented by dark blue dots. The pale purple dots represent the general $CD8^+$ T cell population.

### 3.8.6 WHAT IS THE PROLIFERATIVE CAPACITY OF VACCINE INDUCED T CELLS IN HCV INFECTED PATIENTS?

No HCV-infected vaccine responder developed proliferative T cell responses to vaccine immunogen as judged by $^{3}$[H]thymidine proliferation assays performed either 6 or 26 weeks after boost vaccination. This contrasted significantly with healthy volunteer responses (Arm A) where typically strong proliferation was observed (Figure 3-21).

Figure 3-21; Proliferative capacity of vaccine induced T cells: 3 healthy volunteers compared with 3 HCV infected patients



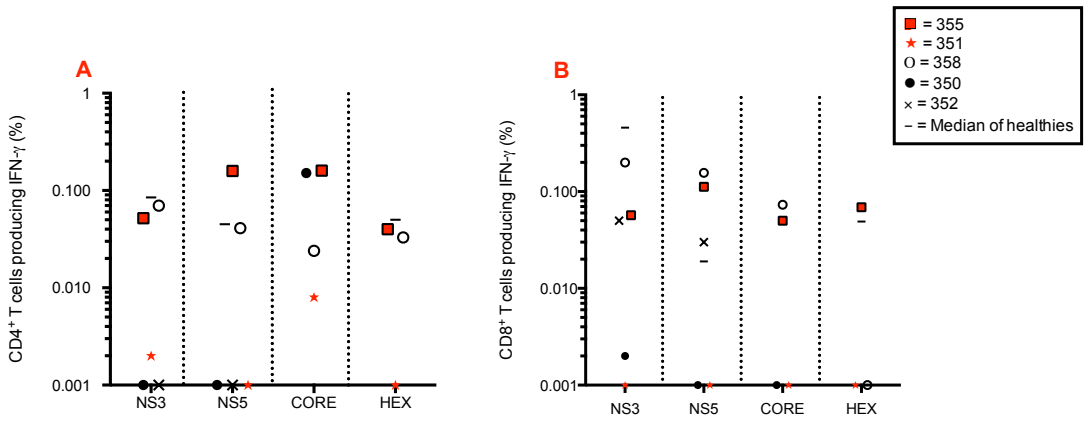

Proliferation in response to HCV antigens included in the vaccine, plotted as Stimulation Index (SI). Wks = week post study commencement. Prime vaccine given at wk 2 and boost at wk 8. Blue columns are 3 representative healthy volunteer vaccine responders, while green columns represent the 3 responding HCV infected patients in Arm C (not on PEG-IFN/ribavirin therapy). Proliferation responses are normalised against unstimulated controls. Only those columns >1.0 are coloured. A positive response was defined as a SI > 3.0 – no HCV infected responders developed proliferative responses defined as positive using this cut-off.

### 3.8.7 WHAT IS THE CYTOKINE PROFILE OF VACCINE INDUCED T CELLS?

The functionality of the antiviral response induced by vaccination was assessed in 5 HCV infected vaccine recipients using intracellular cytokine staining for IFN-γ and TNF-α. Two pools of peptides representing NS3/4 and NS5A/B were used to stimulate PBMCs and compared with responses to HCV core peptides and adenoviral capsid hexon protein (HEX) (see Methods 2.8.3, page 82).

Of the 5 patients assessed, 2 were vaccine-responders as judged by novel IFN-γ ELISpot responses after vaccination to vaccine immunogen (pts 355 and 351) and are shaded in red in Figure 3-22. Amongst the 3 vaccine non-responders, 2 had natural T cell responses against HCV immunogen at baseline (pts 358 and 352), while the final patient (pt 350) had no T cell response detectable by IFN-γ ELISpot at any time-point.

Figure 3-22; IFN-γ production following peptide pool stimulation of PBMCs collected 1 week after boost vaccination

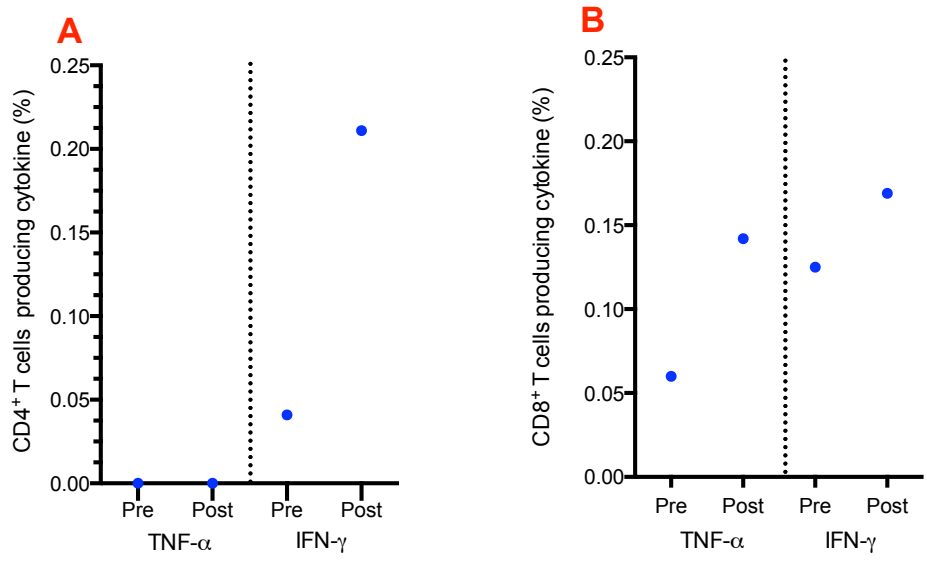



Graph (A): $CD4^+$ T cells. Graph (B): $CD8^+$ T cells. Horizontal dashes represent median values for 10 healthy volunteer vaccine recipients (Arm A). All other points represent individual patients (see legend). Symbols coloured in red represent vaccine responders (as judged by IFN-γ ELISpot assay). Responses to peptide stimulation (using pools representing NS3, NS5, core or HEX) in vitro were measured by intracellular cytokine staining. HEX= Adenovirus hexon protein.

In 1 vaccine responder (Pt. 355) both CD4+ and CD8+ T cells had detectable responses to peptides pools representing NS3 and NS5 (vaccine immunogen) as measured by intracellular cytokine staining for IFN-γ. This patient also had detectable T cell responses to HCV core protein suggesting natural T cell immunity targeting core – this is supported by their baseline IFN-γ ELISpot assay in which T cell responses to peptide pool A (corresponding to HCV core) were detectable (Appendix 7).

Assessment of longitudinal cytokine production by T cells for patient 355 is presented in Figure 3-23. Higher percentages of both CD4+ and CD8+ T cells produced IFN-γ after boost vaccination than after prime vaccination (0.21% vs. 0.04% and 0.17% vs. 0.13% respectively for post vs. pre boost) For CD8+ T cells only, there was an increase in TNF-α producing cells between priming and boosting (0.06% to 0.14% of cells positive).

Figure 3-23; Cytokine production by CD4+ and CD8+ T cells isolated from patient 355 before and after boost vaccination following peptide stimulation in-vitro




Graph (A): CD4+ T cells. Graph (B): CD8+ T cells. The y-axis represents the summed percentage of T cells producing the relevant cytokine after stimulation with either peptide pool NS3-NS4 or NS5A-NS5B. Pre= treatment week 4 (patient 355 had already received prime vaccination at this time-point). Post = 1 week after boost vaccination.

## 3.9 SUMMARY DISCUSSION

This is the first study in man to employ a heterologous prime/boost vaccination regime with adenovirus and MVA vectors against hepatitis C virus infection. Successful and safe induction of anti-HCV T cells by vaccination is demonstrated in HCV infected patients, both during PEG-IFN/ribavirin therapy, and without adjuvant therapy.

I first evaluated baseline anti-HCV T cell responses that occur naturally in the setting of chronic HCV infection using peptides corresponding to the vaccine immunogen (HCV NS genotype 1b) in IFN-γ ELISPOT assays. Prior to vaccination, HCV specific T cell responses were detected in 4 of the 13 patients enrolled in the study. This is in keeping with previously published data reporting the frequency of detectable anti-HCV responses during chronic HCV infection (Kaplan et al. 2005; Lechner et al. 2000; Wedemeyer et al. 2002). Fine mapping of these responses identified 5 immunogenic epitopes, 4 of which have not previously been reported.

Sequencing autologous virus in regions targeted by HCV specific T cells at baseline demonstrated that, in all but 1 instance, circulating virus contained an amino acid sequence that differed from the amino acid sequence of the peptides used in our IFN-γ ELISpot assay (and therefore our vaccine immunogen). Whilst these might represent the selection of escape mutations driven by natural T-cell immunity, these variants might simply reflect the natural diversity of HCV viral sequences that occurs as a consequence of the poor proof reading function of the viral NS5 polymerase.

Next, induction of T cell responses by prime vaccination with ChAd3-NSmut, followed 8 weeks later by boosting with MVA-NSmut was assessed. 11 HCV infected patients received prime/boost vaccination, 7 during PEG-IFN/ribavirin therapy (Arm B) while the

remaining 4 were vaccinated without adjuvant therapy (Arm C). Vaccination successfully induced novel anti-HCV T cell responses in 5 patients. Peak responses were typically detected 2 weeks after boosting (median peak response 275 SFU/$10^6$ PBMCS vs. baseline 32 SFU/$10^6$ PBMCS). Using HCV peptide pools that spanned the entire HCV genome, induction of T cell responses was clearly limited to peptides within the vaccine insert, providing further evidence that these responses were vaccine induced.

Where possible, positive IFN-γ ELISpot responses were next mapped to identify the breadth, and ultimately the individual peptide targets of vaccine induced T cells. The greatest breadth of induced T cell responses was identified in patient 359 who responded to 3 peptide pools, whilst 1 patient (patient 355) responded to 2 pools, and the remaining 3 patients (351, 357 and 360) developed responses to just 1 peptide pool. This contrasted significantly with healthy volunteers enrolled in Arm A of the HCV003 study where 3 volunteers developed T cell responses to all 6 peptide pools, and the average number of pools to which vaccine induced responses were detected was 4 (unpublished data).

Using fine mapping, 4 peptides (15 amino acids in length, 2 of which were overlapping) were identified as targets of vaccine induced T cells. All 4 of these contained immunogenic epitopes that have previously been reported in the setting of natural HCV infection, $NS3_{1406}$, $NS3_{1395}$ and $NS5_{2986}$ (Wedemeyer et al. 2002; H. Wang & Eckels 1999; Guo et al. 2012).

Despite successful induction of anti-HCV T cell responses by vaccination in 3 of the 4 patients in Arm C (who were not on PEG-IFN/ribavirin therapy) there was no significant decrease in circulating viral load in any of these individuals. This finding raised two key questions; i) do vaccine induced T cells recognise autologous circulating virus, and ii) if

vaccine induced T cells recognise virus, what is their capacity to effect killing of infected cells?

To address the first of these questions, autologous virus was sequenced at immunogenic epitopes both at baseline and, when possible, following boost vaccination. In all cases, the circulating viral sequence at baseline in vaccine responders differed from that of the vaccine immunogen. Furthermore, vaccine induced T cells did not target these variants as judged by IFN-γ ELISpot assays using peptides matching viral sequence. In addition, there was no change in viral sequence (at immunogenic epitopes) after vaccination in any of the 3 vaccine responders in Arm C, further suggesting that vaccine induced T cells do not exert mutagenic pressure on circulating virus.

These findings highlight the importance of comparing autologous viral sequences to vaccine immunogen in therapeutic HCV vaccine studies. Clearly, successful induction of T cells by vaccination serves no therapeutic purpose, if primed T cells cannot recognise autologous virus. To date, all published therapeutic vaccine studies for HCV have not made this important comparison (see Section 1.3.2, page 43). Most strikingly, a phase 1 study evaluating the therapeutic vaccine TG4040 (an MVA vector that expresses NS3/4/5B proteins) has progressed to a phase 2 study without making this important comparison (Habersetzer et al. 2011; Wedemeyer et al. 2012). Although minor changes in viral load were observed after vaccination in their original study, these might be explained by natural variation during chronic infection, rather than successful induction of T cells that appropriately target virus.

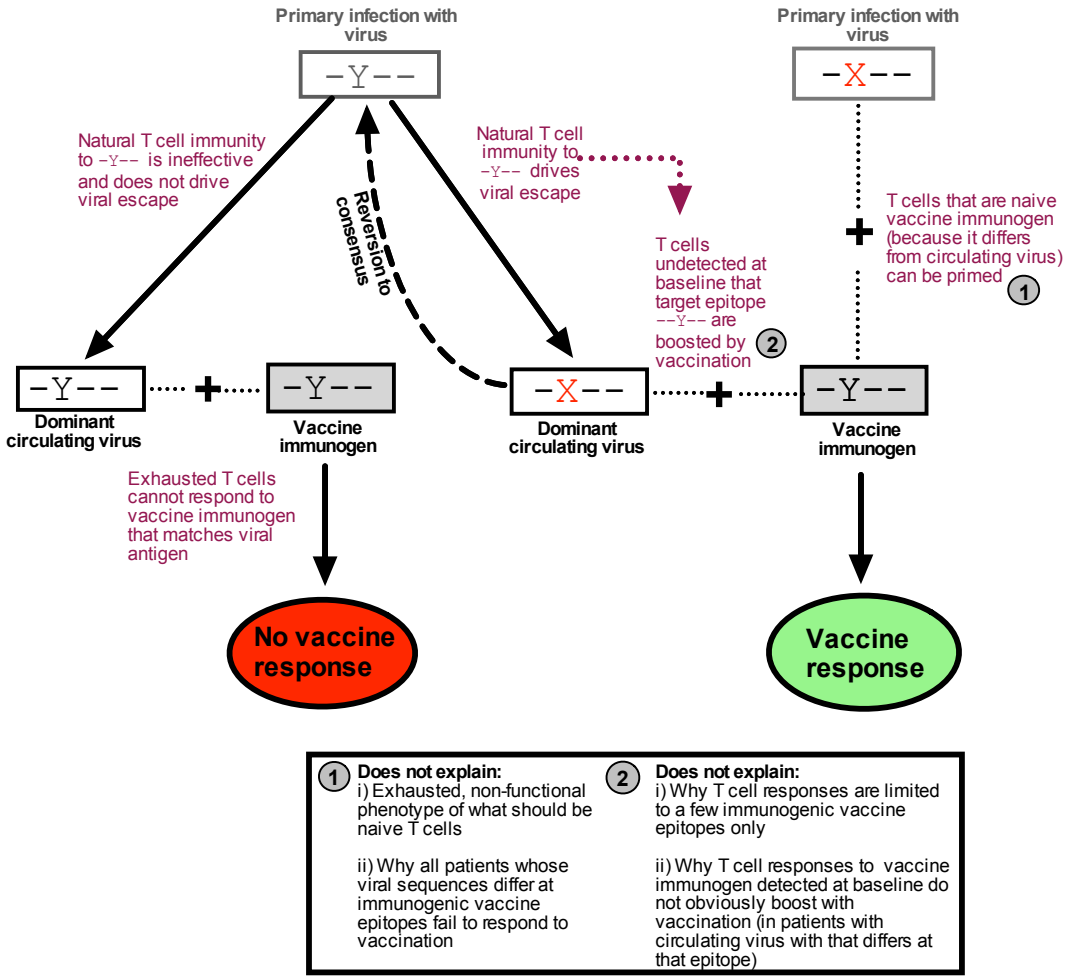
One simple explanation for the difference between circulating virus and vaccine sequence at immunogenic epitopes is that, in these regions, the vaccine may represent an

uncommon HCV sequence. This explanation was supported for 2 epitopes ($NS3_{1406}$ and $NS5B_{2984}$) by evaluating sequence diversity across all genotype 1 HCV sequences available on the Los Alamos HCV database. For $NS3_{1406}$, only 4.1% of 809 genotype 1b, and none of the 839 genotype 1a sequences analysed matched vaccine immunogen. In fact, the virus was highly diverse at this epitope, with no variant present in more than 25% of sequences reviewed. Similarly, $NS5B_{2984}$ was an infrequent variant amongst genotype 1a and 1b HCV viruses (0.2% and 31.8% of populations respectively).

Natural sequence diversity may also explain the finding of a viral sequence that differed from vaccine epitope $NS3_{1395}$ in, and isolated to, patient 359 (Table 3-5). However, this HLA-B8 restricted epitope is highly conserved (87.5% of all genotype 1 sequences on the Los Alamos database) and patient 359 was 1 of 3 HLA-$B8^+$ patients enrolled, suggesting the possibility that historical immune pressure may have led to an escape variant. Interestingly, although no T cells targeting this epitope were detectable prior to vaccination, patient 359 was the only HLA-$B8^+$ vaccine recipient who developed T cell responses to this epitope after vaccination. A similar observation was made in the HCV002 trial, where out of 6 HLA-$B8^+$ vaccine recipients, the only patient who responded to epitope $NS3_{1395}$ was also 1 of only 2 patients whose circulating virus contained a variant amino acid sequence at the epitope (unpublished data).

Apart from viral sequence diversity, what other explanations are there for the reason why autologous viral sequence always differs at vaccine induced T cell epitopes from vaccine immunogen (Figure 3-24)?

Figure 3-24 Why do vaccine immunogen and viral sequence always differ at immunogenic epitopes in HCV infected vaccine responders?




'–x––' and '–y––' represent either the amino acid sequence of vaccine immunogen or the corresponding viral amino acid sequence as indicated (normally 9-12 amino acid in length peptides). For the purpose of this flow diagram, these 2 sequences are assumed not to be cross-recognised by T cells.

Let us first consider vaccine non-response at immunogenic epitopes in patients whose viral sequence matches vaccine immunogen – this might be explained by exhaustion, whereby as a consequence of chronic viral antigen exposure, antigen specific T cells have developed an 'exhausted phenotype' meaning they proliferate poorly, have impaired

production of antiviral cytokines, and respond poorly to vaccination (see Section 1.2.2(ii), page 35) (Barber et al. 2005; Missale et al. 2012). For this to explain vaccine non-response in HCV003 patients, we must also assume that our IFN-γ ELISpot is insensitive for the detection of exhausted HCV specific T cell at baseline. Evidence to support this assumption comes from a 2002 study by Lauer and colleagues that demonstrated in-vitro stimulation of PBMCs isolated from HCV infected patients can reveal subdominant HCV specific T cells that are not detected by IFN-γ ELISpot assay (using HCV peptide pools similar to our own)(Lauer et al. 2002).

Accepting that our IFN-γ ELISpot does not detect all circulating HCV specific T cells also provides one potential explanation why autologous viral sequence at vaccine induced T cell epitopes always differs from vaccine immunogen. It may be that T cells defined as 'vaccine induced' are actually present at baseline, but at a frequency that is too low to detect by IFN-γ ELISpot. If this is true, these cells may have historically exerted mutagenic pressure on circulating virus, thereby leading to escape variants that differ from vaccine immunogen. Furthermore, these HCV specific cells will only rarely encounter antigen (when circulating virus reverts to its original consensus sequence during replication) and will therefore not present a phenotype that is 'as exhausted' as T cells that are persistently presented with high antigen loads. When vaccination presents these memory T cells with an appropriate antigen load, they will therefore effectively 'boost' to levels detectable by ELISpot. Work is underway to evaluate this possibility.

Even if T cells that recognise autologous virus can be induced by vaccination, these cells may have impaired function and be unable to generate an effective immune response against infected cells. For this reason, using HLA class I matched pentamers to epitope $NS3_{1406}$ the phenotype of vaccine induced T cells was assessed in 3 patients at their peak

response following boost vaccination. Vaccine induced T cells were predominantly effector memory T cells that were high in granzyme A and B, and perforin. Vaccine induced T cells frequently express PD-1, a marker typically indicative of cell exhaustion (Barber et al. 2005). However, PD-1 is also a marker of cell activation and notably, other activation markers (CD38 and HLA-DR) were also high on vaccine induced T cells (Kasprowicz et al. 2008). Nevertheless, this raises some concern that even if T cells targeting viral epitopes can be induced, they might be impaired in their cytolytic and other effector functions.

An important comparison should be drawn between HCV infected patients and the healthy volunteers in Arm A of the trial. Clearly, patients with chronic HCV infection display impaired vaccine responses as judged by the frequency, breadth and magnitude of IFN-γ ELISpot responses, as well as T cell proliferation responses when compared to healthy volunteers. Why should this be the case? Firstly, chronic HCV infection itself may impair priming of naïve T cells through a variety of mechanisms including; altering the cytokine milieu in which T cells are primed and increasing circulating regulatory T cells (see Section 1.2.2 (page 35) for further detail). Furthermore, for patients who received vaccination during therapy, the pleiotropic immunomodulatory effects of PEG-IFN may have impaired T cell priming (Rizza et al. 2011; Launay et al. 2008). It is also possible that in HCV infected individuals (unlike healthy volunteers), vaccine induced T cells might be sequestered in the liver where they target infected cells – in this setting IFN-γ ELISpot assays based on circulating T cell responses might not accurately reflect the true response to vaccination.

Although numbers are small, the finding that 75% (3 of 4) of HCV infected patients who received vaccination alone, compared with only 29% (2 of 7) of those vaccinated during

PEG-IFN therapy developed novel T cell responses suggests the possibility that therapy may impair vaccine responses (p=0.24, Fischer's exact). Clearly, for an effective therapeutic vaccination strategy to be carried forward it is important to have a detailed understanding of the effects of PEG-IFN/ribavirin therapy on immune responses – this forms the basis of the second investigative chapter of this thesis.

## 3.10 CONCLUSION

This is the first study to successfully demonstrate safe induction of anti-HCV T cells by vaccination using a combination of adenovirus and MVA based vaccines. Responses to vaccination are abrogated in patients with chronic HCV infection compared to healthy volunteers as measured by the frequency of T cell induction, and also the magnitude and breadth of these responses.

As a potential therapy for chronic HCV infection, several important issues relating to appropriate T cell induction by vector based vaccines are raised. In all cases where vaccination induced novel T cell responses, these did not recognise autologous virus. Furthermore, where good matches between autologous virus and vaccine immunogen existed, T cells targeting these regions were not generated. These findings highlight the importance of interpreting vaccine induced T cells in the context of the autologous viral sequence in therapeutic vaccine studies. They also raise the possibility that vaccine may be unable to prime T cells that target circulating virus because these cells are already antigen experienced and have an exhausted phenotype.

A successful therapeutic vaccine will need to contend with these issues and work is underway to identify optimal epitope targets to better inform future vaccine designs.

Furthermore, the impact of concurrent PEG-IFN/ribavirin therapy on immune cell subsets, and the potential impact this may have on vaccine responses is poorly understood and warrants further investigation. This will be explored further in the following chapter.

# Chapter 4 The Host Immune Response to Standard Therapy during chronic Hepatitis C virus infection (THIRST trial)

## 4.1 INTRODUCTION

This chapter stems from the finding of reduced immunogenicity of ChAd3-NSmut/MVA-NSmut vaccination in HCV infected patients compared with healthy volunteers in the HCV003 trial, particularly those receiving concurrent PEG-IFN/ribavirin therapy. In order to establish potential reasons for this finding, this chapter broadly investigates baseline differences in lymphocyte subpopulations during HCV infection and the immunomodulatory effect of PEG-IFN/ribavirin therapy. Please see Chapter 1 (Section 1.6, page 67) for further background relating to this trial (THIRST trial).

## 4.2 PRIMARY HYPOTHESES

Therapeutic vaccination in HCV infected patients fails to induce T-cell responses because:

1. Chronic HCV infection impairs host immune function and therefore the capacity to generate vaccine responses.
2. PEG-IFN/ribavirin therapy administered concurrently with vaccination has negative immunomodulatory effects that impair T-cell priming.

## 4.3 PRIMARY OBJECTIVES

1. To identify baseline functional, phenotypic and differentiation status differences in immune cell subpopulations between healthy individuals and patients with chronic HCV infection.
2. To evaluate the impact of PEG-IFN/ribavirin longitudinally on the function, phenotype and differentiation of immune cell subpopulations

## 4.4 KEY QUESTIONS

Does chronic HCV infection or PEG-IFN/ribavirin therapy change the phenotype or distribution of subsets of circulating immune cell populations?

Specifically, I will address whether chronic HCV infection or PEG-IFN/ribavirin therapy causes changes in circulating lymphocyte:

1. Subpopulations
2. Cytotoxic capacity
3. Homing phenotype
4. Function
5. Activation status
6. Differentiation/memory status

This chapter diverges slightly from the traditional approach of presenting all investigative results followed by a summary discussion. Instead, results pertaining to each of these questions will be accompanied by a brief, but separate discussion. The aim of this approach is help present the large volume of phenotypic data gathered in a clearer manner.

# 4.5 DEMOGRAPHICS AND CLINICAL DATA

## 4.5.1 HCV INFECTED PATIENTS ENROLLED IN THIRST TRIAL

### *Baseline data*

Thirteen patients with a mean age of 42.4 years were prospectively enrolled in the THIRST trial between January 2011 and June 2011 (Table 4-1).

**Table 4-1; THIRST trial baseline patient demographics**

| Patient no. | Age | Gender | Ethnicity | HCV Geno-type | Fibrosis score | Duration of infection (years) | Viral load (IU/ml) | ALT (IU/L) | IL-28 host genotype |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 37 | M | Caucasian | 4 | F 1-2/6 | 4 | 367,000 | 59 | T/T |
| 2 | 30 | F | Caucasian | 3a | Unknown | 5 | 214,000 | 47 | T/T |
| 3 | 28 | F | Caucasian | 3a | Unknown | 1.5 | 273,000 | 42 | T/G |
| 4 | 43 | M | Caucasian | 1 | F 2/6 | > 20 | $16\times10^6$ | 23 | T/T |
| 5 | 41 | M | Asian | 3a | Unknown | 10 | $2.1\times10^6$ | 72 | T/G |
| 6 | 32 | M | Asian | 4 | Unknown | > 5 | 492,000 | 82 | T/T |
| 7 | 49 | M | Caucasian | 3 | F 5/6 | > 20 | $2.7\times10^6$ | 151 | T/T |
| 8 | 41 | M | Caucasian | 3a | Unknown | Unknown | 73,000 | 25 | T/G |
| 9 | 61 | F | Caucasian | 3 | Unknown | > 10 | $1.9\times10^6$ | 144 | T/G |
| 10 | 56 | F | Caucasian | 3 | Unknown | Unknown | 590,000 | 47 | T/G |
| 11 | 48 | M | Caucasian | 3 | F4/6 | 26 | 40,500 | 149 | T/T |
| 12 | 36 | F | Caucasian | 3 | Unknown | 6 | 128,000 | 13 | G/G |
| 13 | 50 | F | Caucasian | 3 | Unknown | 10 | 898,000 | 64 | T/T |
| | 42.4 Mean | 6/13 F | | | | | | 8/13 ↑ALT | |

IL28 genotype is based on the single nucleotide polymorphism (SNP) rs80999917 where T/T= protective allele. Where duration of infection is listed as unknown, patients had evidence of HCV infection >1 year. All fibrosis scores relate to liver biopsies taken within 2 years of study enrolment and are graded by Ishak fibrosis score. F=female. M=male.

Of the patients enrolled, 10/13 were infected with genotype 3 HCV. Only patient 7 (Pt. 7) was known to be cirrhotic, while the remainder had either no clinical or biochemical parameters to suggest cirrhosis or a biopsy proving otherwise. 6 patients were T/T

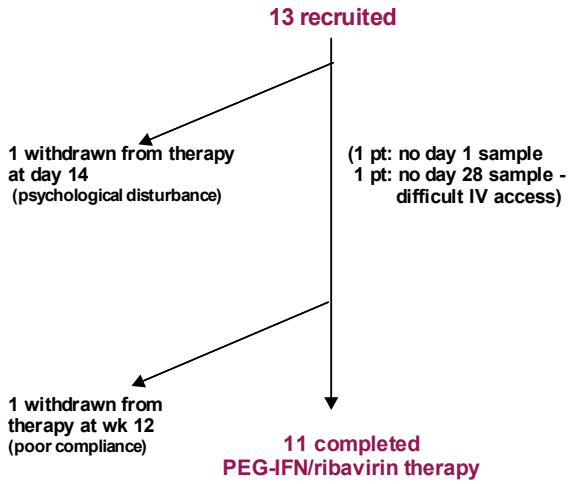
homozygous for the IL28 genotype (SNP rs80999917), 5 were heterozygous (T/G) and the remaining 2 were G/G homozygous.

### *Clinical progress during PEG-IFN/ribavirin therapy*

Of the 13 patients who commenced PEG-IFN/ribavirin therapy, 11 completed the full treatment course (Figure 4-1). Patients with genotype 3 HCV infection received 6 months treatment (apart from patient 7 who received 12 months therapy in keeping with current treatment guidelines for cirrhosis). During therapy, this patient's PEG-IFN dose was reduced temporarily on 2 occasions (both after week 8) due to thrombocytopenia. Genotype 1 or 4 HCV infected patients received 12 months treatment.

1 patient (Pt. 8) ceased PEG-IFN/ribavirin after only 2 weeks due to psychological disturbance. A 2nd patient (Pt. 6) was withdrawn from treatment after 12 weeks due to poor treatment compliance. A blood sample on day 1 was not collected for 1 patient who successfully completed HCV therapy. In all, a complete set of blood samples (day 0, 1, 7 and 28) for the THIRST trial was obtained from 10 of the 13 patients enrolled. Where possible, patients who had an incomplete set of blood samples were still included in the study analysis.

Figure 4-1; THIRST trial recruitment and clinical progress during therapy




Pt = patient; Wk = week

### *How did circulating lymphocyte count change during therapy?*

As expected based on previous data during the first 3 months of PEG-IFN/ribavirin therapy there was a progressive decrease in circulating lymphocyte counts, followed by a relative plateau (Table 4-2) (Humphreys et al. 2012).

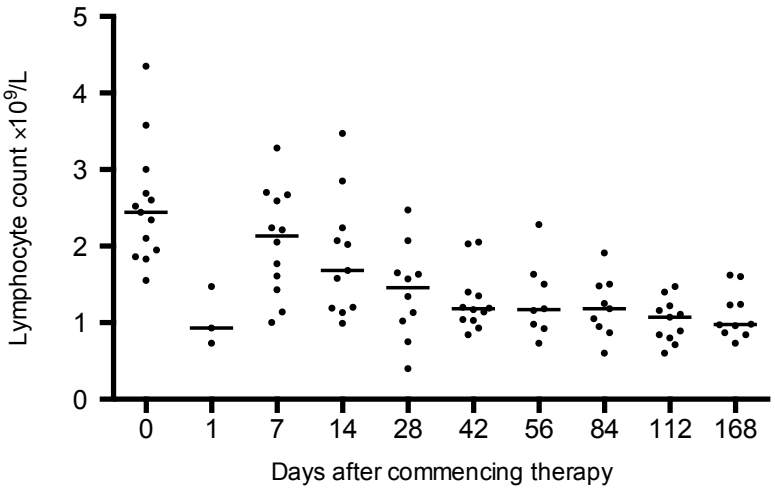
Table 4-2; Circulating lymphocyte counts during PEG-IFN therapy

| | Lymphocyte count ($\times 10^9$/ml) | Compared with day 0 |
|---|---|---|
| Day 0 | 2.28 | |
| Day 1 | 1.10 | p=0.25 (only 3 samples) |
| Day 7 | 1.77 | p=0.73 |
| Day 14 | 1.39 | p = 0.03 |
| Day 28 | 1.18 | p < 0.01 |
| Day 56 | 1.17 | p< 0.01 |
| Day 84 | 1.18 | p< 0.01 |
| Day 112 | 1.07 | p< 0.01 |
| Day 168 | 0.98 | p< 0.01 |

Median lymphocyte counts at each time-point are given. p values are calculated using Wilcoxon signed ranked test (for non-parametric, paired samples)

In the original study design, day 1 blood samples were collected for assessment of the phenotype of PBMCs only. Day 1 full blood counts (FBCs) were not routinely collected and only assessed in 3 individuals. For these 3 patients a transient but substantial decrease in lymphocyte count at this early time-point was observed (Figure 4-2).

Figure 4-2; Longitudinal circulating lymphocyte counts during PEG-IFN/ribavirin therapy in HCV infected patients

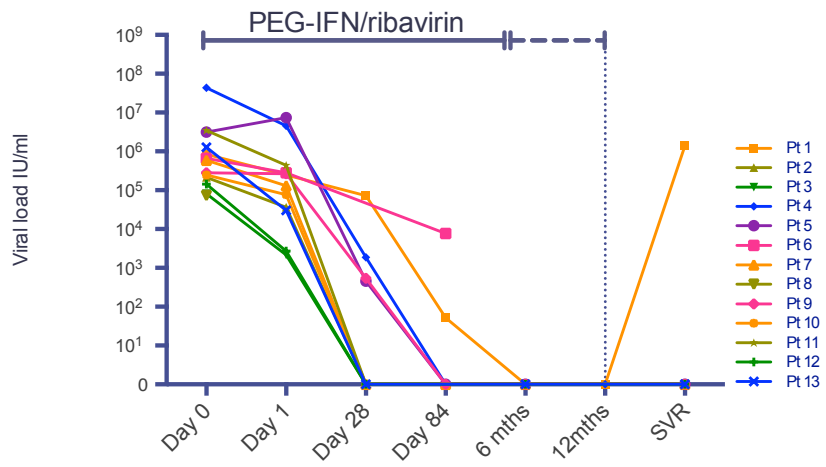



Each point represents the total circulating lymphocyte count in an individual patient at the relevant time-point. Horizontal lines depict median values at each time-point.

### *How did viral load change during therapy?*

Circulating viral loads measured during therapy are demonstrated in Figure 4-3. Between day 0 and day 1 of therapy there was a significant decrease in viral load from a median of $4.2\times10^6$ IU/ml to $1.2\times10^6$ IU/ml ($p = 0.04$). Six patients achieved an RVR (undetectable virus at week 4): patients 2, 3, 7, 10, 11, 12 and 13. All 11 patients who completed treatment were virus negative at the end of therapy. 1 of these, patient (1) relapsed during follow-up, while the remainder achieved an SVR.

Figure 4-3; HCV viral kinetics during PEG-IFN/ribavirin therapy




The x-axis is the time from the start of PEG-IFN/ribavirin treatment. Patients either received 6 or 12 months (indicated by horizontal dashed line) therapy depending on HCV genotype and whether they were cirrhotic. The y-axis is circulating viral load (log scale). Coloured lines connect individual patients according to their viral load over time (see legend).

### 4.5.2 HEALTHY VOLUNTEERS

A group of 12 healthy controls was recruited with a mean age of 40.8 (Table 4-3) for comparison with HCV infected patients at baseline. These individuals had a similar gender and age distribution to HCV infected patients recruited to the THIRST trial, but were not individually matched (healthy control mean age 40.8 years vs. patient mean age 42.4 years, p = 0.71, student's t-test).

Table 4-3; Healthy control demographics for volunteers enrolled in the THIRST study

| Control no. | Age (yrs) | Gender | Ethnicity |
|---|---|---|---|
| 1 | 33 | M | Caucasian |
| 2 | 32 | M | Asian |
| 3 | 26 | F | Caucasian |
| 4 | 34 | M | Caucasian |
| 5 | 35 | M | Caucasian |
| 6 | 31 | F | Caucasian |
| 7 | 37 | M | Caucasian |
| 8 | 56 | F | Caucasian |
| 9 | 41 | F | Caucasian |
| 10 | 61 | F | Caucasian |
| 11 | 57 | F | Caucasian |
| 12 | 47 | F | Caucasian |
| | 40.8 (Mean) | 7/12 female | |

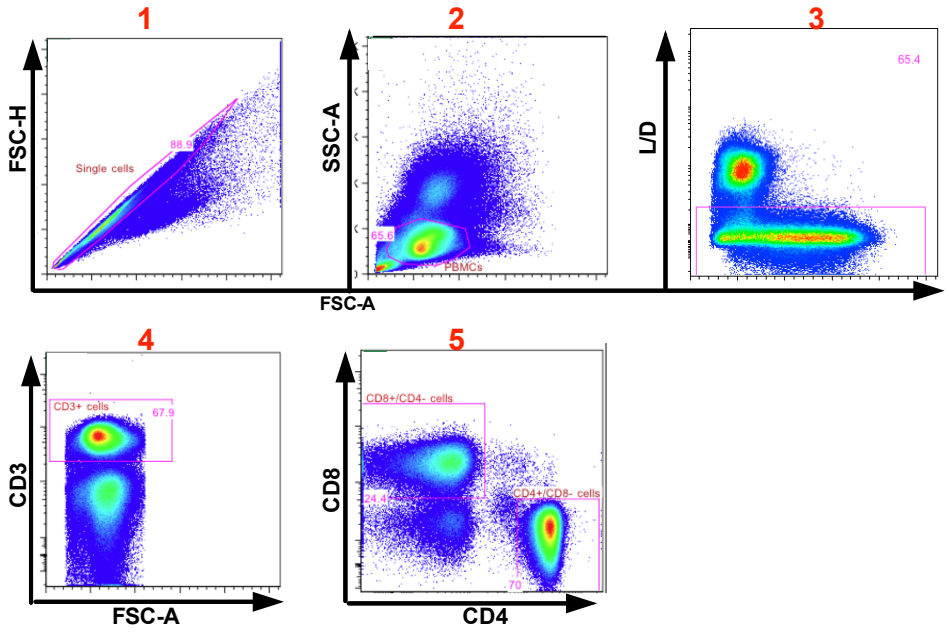
## 4.6 KEY QUESTION 1 - Does HCV infection or PEG-IFN/ribavirin therapy change lymphocyte subsets in circulation?

The lymphocyte populations assessed in this section were CD3+ lymphocytes, CD4+ and CD8+ T cells, B cells, regulatory T cells, CD161+ T cells and NK cells.

### 4.6.1 CD3+, CD4+ AND CD8+ T CELLS

An example of the typical FACS gating strategy used to define CD3+, CD4+ and CD8+ T cells is provided in Figure 4-4. Between individuals, gating position was occasionally adjusted to correct for inter-individual variability, but otherwise gating was kept constant across time.

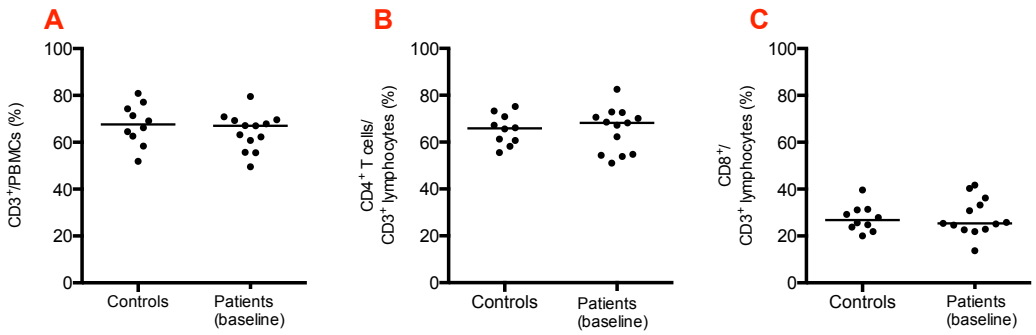
Figure 4-4; FACS gating strategy used to define CD3+, CD4+ and CD8+ T cells




Samples were first gated on single cells (1), followed by PBMCs (2) and then live cells (3) before defining CD3+ (4) and CD4/CD8+ T cells (5). FSC-H: Forward scatter-height; SSC-A: Side scatter area; FSC-A: Forward scatter area; L/D: Live/dead marker

At baseline, there was no significant difference in the proportion of circulating CD3+ PBMCs, CD4+ or CD8+ T cells between patients with CHC and healthy controls (Figure 4-5).

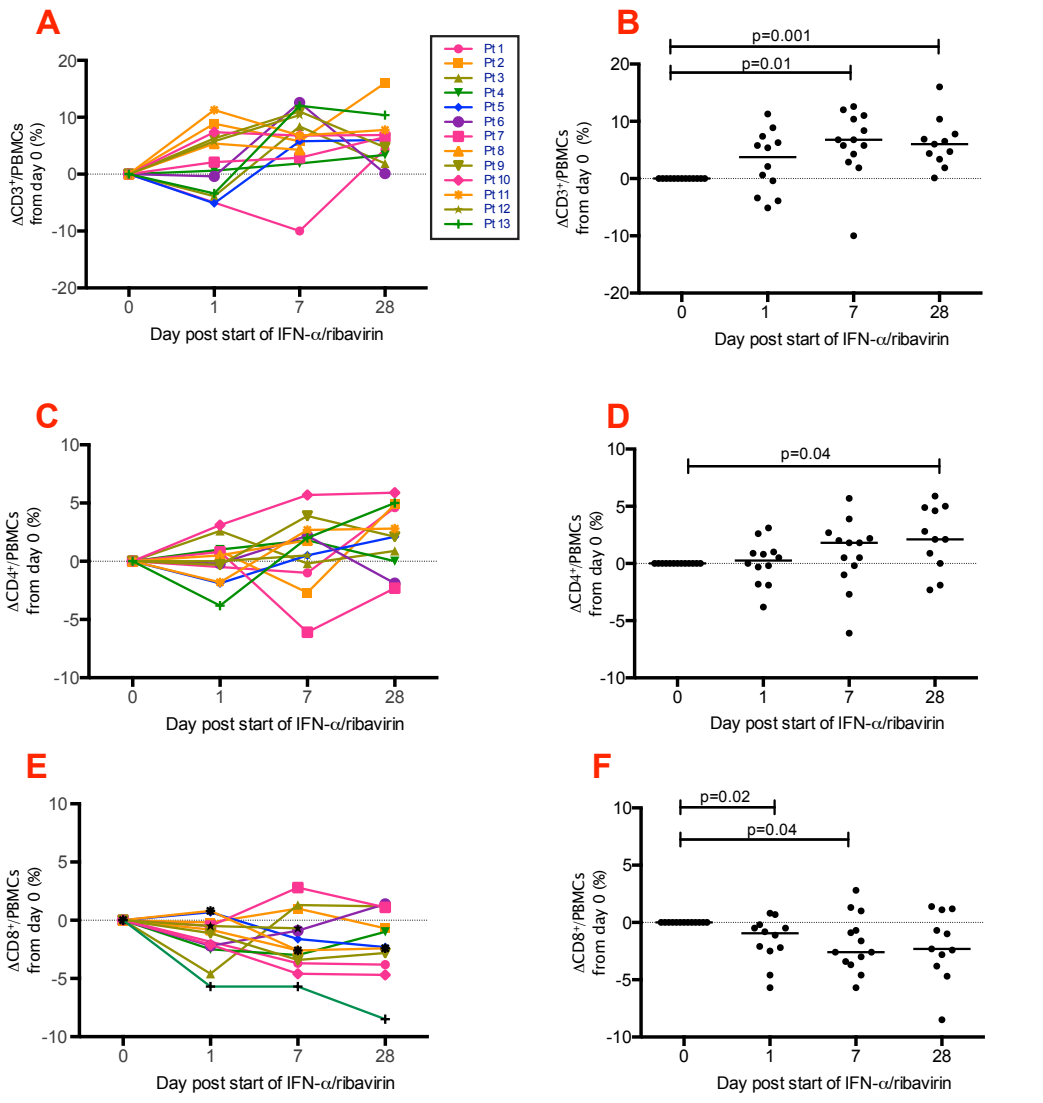
Figure 4-5; CD3+, CD4+ and CD8+ T cell populations in patients with CHC compared with healthy controls




Graph (A): $CD3^+$ cells as proportions of PBMCs; Graph (B): $CD4^+$; and Graph (C): $CD8^+$ populations as proportions of $CD3^+$ T cells. Horizontal lines depict group medians. Samples for patients were taken at baseline prior to therapy

Between day 0 and day 1 of therapy, CD8+ T cells decreased by a median of 1.0% (p = 0.02) (Figure 4-6). Over 28 days of therapy, CD3+ cells increased by a median of 6.0% (p < 0.01), while CD4+ T cells increased by 2.1% (p = 0.04) and CD8+ T cells decreased 2.3% (p = 0.07) (all as proportions of PBMCs).

Figure 4-6; Longitudinal changes in circulating lymphocyte subpopulations during PEG-IFN/ribavirin therapy

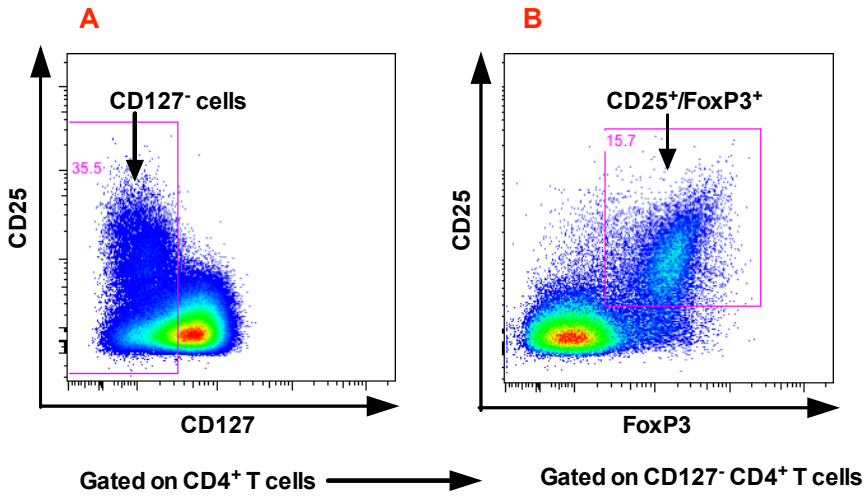



The top row depicts $CD3^+$ lymphocytes, the middle row depicts $CD4^+$ T cells and the bottom row depicts $CD8^+$ T cells, as proportions of PBMCs according to their absolute change from baseline. Left column: Coloured lines connect individual patient samples across time. Right column: Horizontal lines depict group medians. p values are calculated using Wilcoxon signed rank test for paired samples.

### 4.6.2 REGULATORY T CELLS

Traditionally, Foxp3 and CD25 have been used to define regulatory T cells (Tregs) in humans but more recently it has been shown that expression of both these markers can be induced on naïve $CD4^+$ T cells through activation (Gavin et al. 2006). Lack of surface CD127 (IL-7 receptor–α chain) has also been shown to be useful for the delineation of Treg cells: $CD4^+$ T cells that express low levels of CD127 have increased FoxP3 expression and suppressive ability. Therefore, the combination of $CD127^{low}/CD25^+/FoxP3^+$ $CD4^+$ T cells is currently recommended as one of the best methods for defining Tregs with suppressive function (Sakaguchi et al. 2010). This strategy was applied as demonstrated in Figure 4-7.

Figure 4-7; FACS plots demonstrating gating strategy used to define regulatory T cells

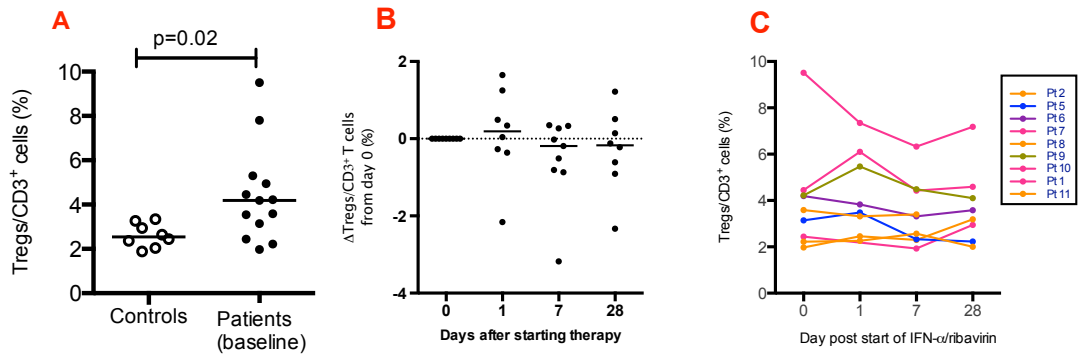



Tregs were defined by first gating on $CD4^+$ T cells, followed by gating on (A) CD127- cells, and then (B) $FoxP3^+/CD25^+$ cells

Baseline circulating Tregs were more frequent in patients with CHC than controls. (4.2% vs. 2.5% of $CD3^+$ lymphocytes, median values, $p = 0.02$). During therapy, Treg changes were not characterised for 4 patients due to difficulties with FoxP3 antibody staining.

However, there was no significant change (or trend) in the proportion of circulating Tregs during the first four weeks of therapy (Figure 4-8).

Figure 4-8; Baseline differences and longitudinal changes in Tregs during HCV therapy




Graph (A): Controls vs. patients at baseline. Graph (B): Values are normalised against day 0 values for each individual patient to present longitudinal change from baseline. Graph (C): Coloured lines connect individual patient samples across time. Horizontal lines depict group medians.

### 4.6.3 B CELLS

The B cell population was defined as shown in Figure 4-9 by gating on $CD19^+$, $CD3^-$ PBMCs.

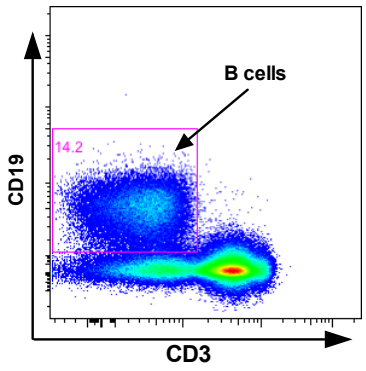
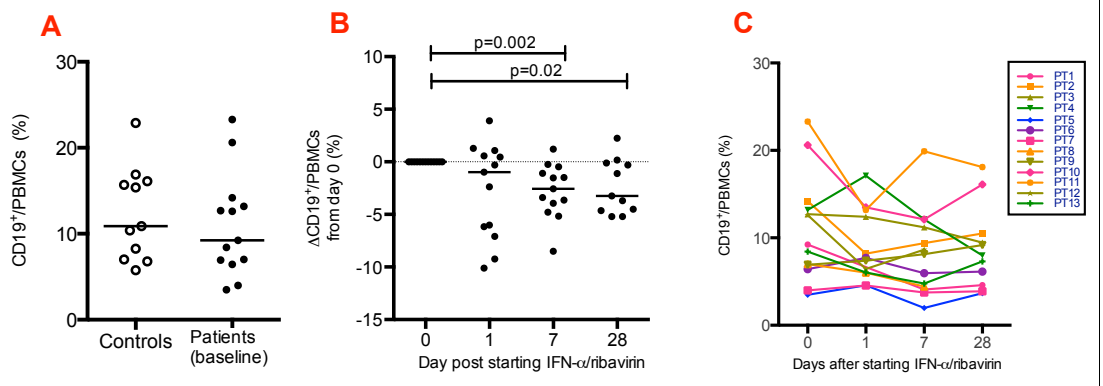



Figure 4-9; Example FACS plot for gating strategy used to define B cells

The B cell population is defined by first gating on live PBMCs followed by $CD19^+$ $CD3^-$ cells.

At baseline, there was no difference in the proportion of PBMCs that were B cells in patients with CHC compared with healthy controls (10.9% vs. 9.2%, p = 0.46, Mann Whitney test) (Figure 4-10). B cells decreased progressively during therapy and had fallen by medians of 2.6% and 3.3% from baseline at day 7 and day 28, respectively (p = 0.002 and p = 0.02).

Figure 4-10; Baseline differences and longitudinal changes in B cells during HCV therapy




Graph (A): Controls vs. patients at baseline. Graph (B): Values are normalised against day 0 values for each individual patient to present longitudinal change from baseline. Graph (C): coloured lines connect individual patient samples across time. Horizontal lines depict group medians

### 4.6.4 CD161$^{+}$ T CELLS

The gating strategy used to define CD161$^{+}$ T cells is demonstrated in Figure 4-11.

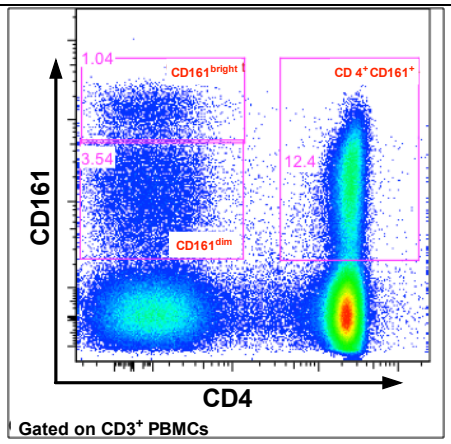



Figure 4-11; FACS plot demonstrating gating strategy used to define CD161$^{+}$ cells

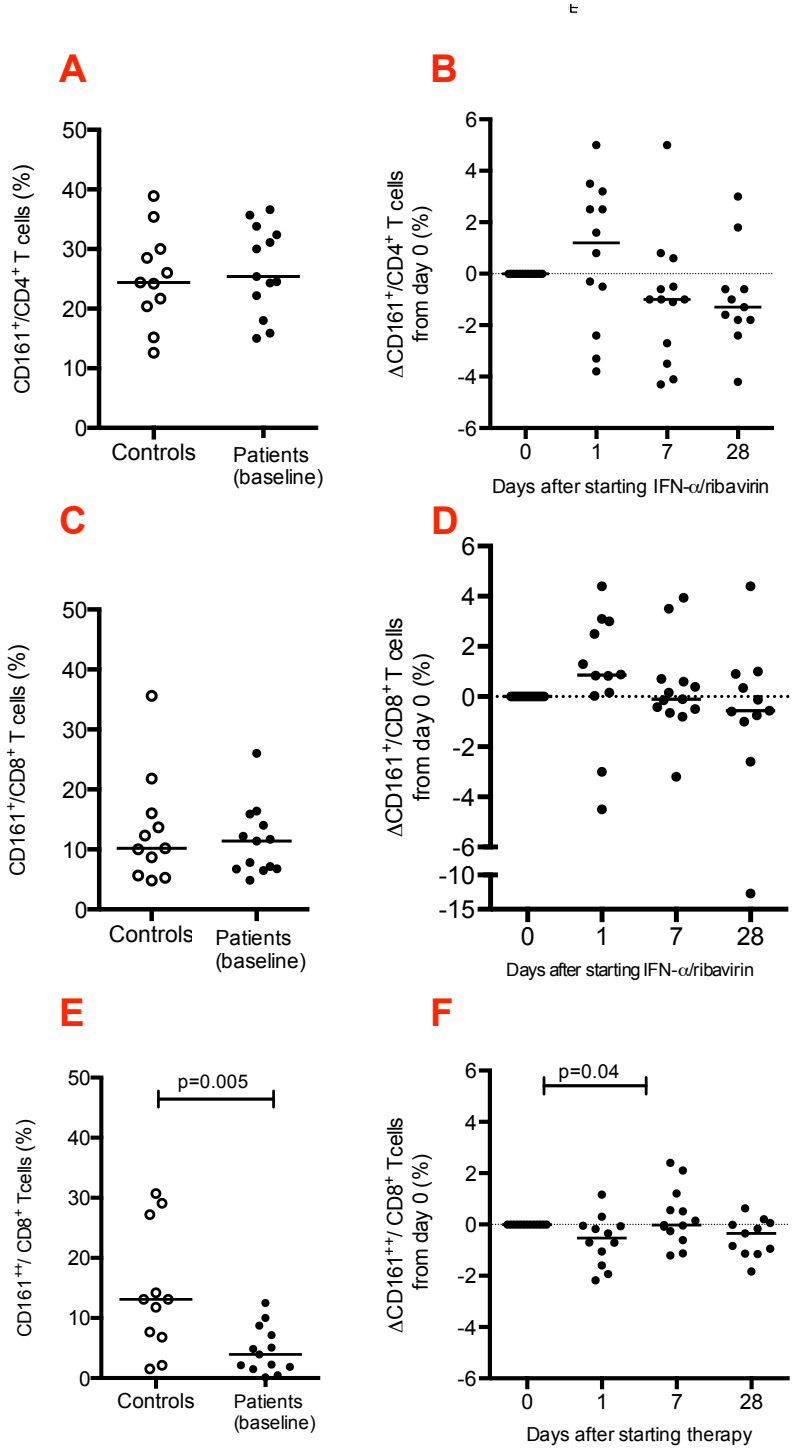
CD4$^{+}$ T cells are defined as CD161$^{+}$ or CD161$^{-}$. CD8$^{+}$ T cells (represented here as CD4$^{-}$ cells are defined as CD161$^{bright}$, CD161$^{dim}$ and CD161$^{-}$ CD8$^{+}$ T cell subsets. This plot has first been gated on CD3$^{+}$ PBMCs.

In patients with CHC, circulating CD8$^{+}$ CD161$^{bright}$ T cells represented only 4.0% of the CD8$^{+}$ T cell population (Figure 4-12-A). This was substantially less than the healthy control population where CD161$^{bright}$ CD8$^{+}$ T cells constituted a median of 13.1% of CD8$^{+}$ T cells ($p = 0.005$). This difference is consistent with previously published data (Billerbeck et al. 2010).

Between day 0 and day 1 of therapy, a small but significant decline in the proportion of CD161$^{bright}$ CD8$^{+}$ T cells occurred, (-0.53% of CD8$^{+}$ cells, median change, $p = 0.04$) (Figure 4-12-B). By day 28, the CD161$^{bright}$ population was still 0.35% less than day 0, although this was not significant ($p = 0.07$), likely in part because a smaller number of patient samples were available for analysis at this time-point (3/13 patients had withdrawn). Graphs depicting longitudinal changes (raw data) for individual patients are included in Appendix 10.

For CD4$^{+}$ T cells there was no significant baseline difference or treatment induced change in CD161$^{+}$ expressing subsets (Figure 4-12).

Figure 4-12; Baseline differences and longitudinal changes during HCV therapy in CD161+ CD4+ and CD8+ T cells

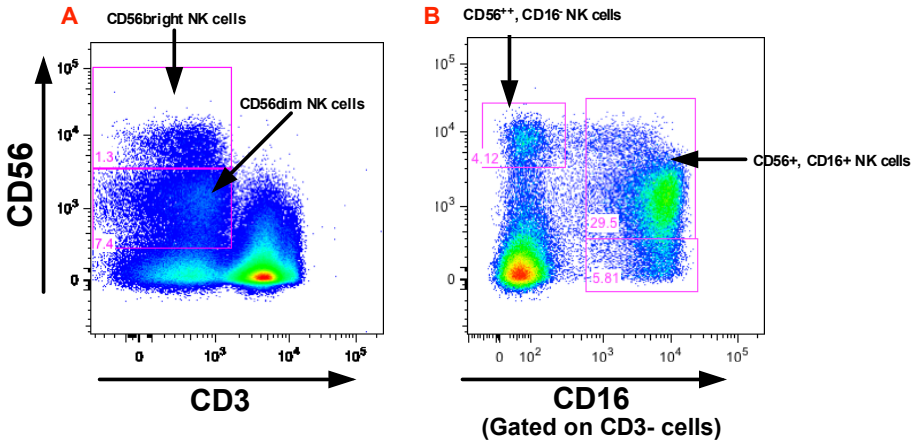



Graphs (A &B): $CD4^+$ $CD161^+$ cells; Graphs (C&D): $CD8^+$ $CD161^+$ (dim) cells; Graphs (E&F) $CD8^+$ $CD161^{++}$ (bright) cells. Graphs in the right hand column present change from baseline during therapy (values are normalised against the day 0 sample). Horizontal lines depict group medians.

### 4.6.5 NK CELLS

Historically, NK cells have been defined for study in a variety of ways depending on surface expression of CD3, CD56 and CD16. For the purpose of the THIRST study, 2 methods were used as outlined in Figure 4-13. After gating on single/live PBMCs, $CD56^{bright}$ and $CD56^{dim}$ NK cells were defined as shown in Figure 4-13-A. An alternative strategy to delineate NK cell subpopulations was also applied (Figure 4-13-B) by gating on $CD3^-$ PBMCs, and then using CD16 to define $CD56^+/CD16^+$ NK, and $CD56^{++}/CD16^-$ NK cells.

Figure 4-13; FACS plots demonstrating gating strategies used to define NK cell subsets




(A) Typical strategy where NK cells are defined as $CD56^{bright}$ and $CD56^{dim}$, $CD3^-$ PBMCs (B) Second strategy applied using CD16 as an additional marker for further delineation of subsets

There was no difference in the NK cell population as a proportion of PBMCs between HCV infected patients and healthy controls (Figure 4-14 and Figure 4-16).

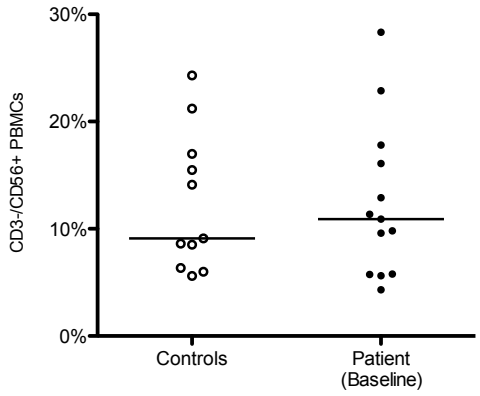
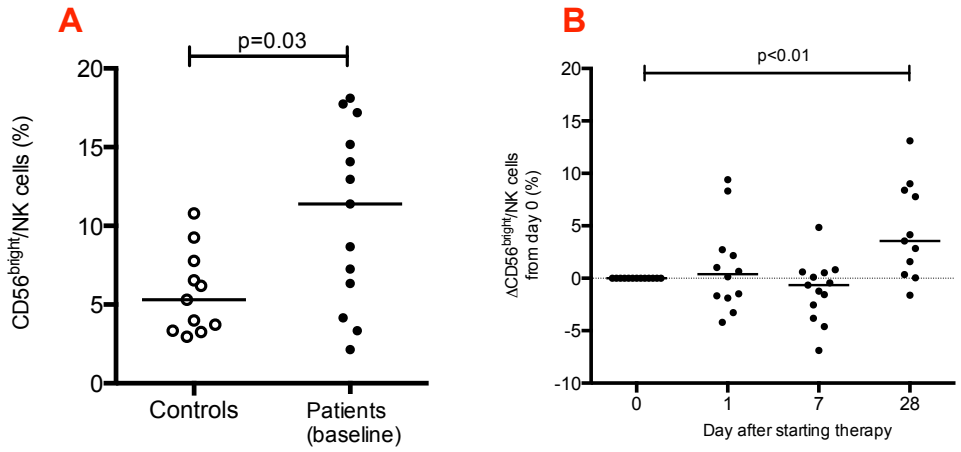



Figure 4-14; Baseline comparison of total NK cell populations between patients and controls

The $CD56^{bright}$ and $CD56^{dim}$ populations have been combined (and evaluated as proportions of circulating $CD3^-$ PBMCs). HCV infected patients on day 0. Horizontal lines depict group medians.

Examining NK cells according to their relative subpopulations (bright vs. dim), I found that $CD56^{bright}$ NK cells were increased in HCV infected patients (11.4%) compared with 5.3% in healthy controls ($p = 0.03$) (Figure 4-15). In addition, over 28 days of therapy this subset increased by a median of 3.6% ($p < 0.01$).

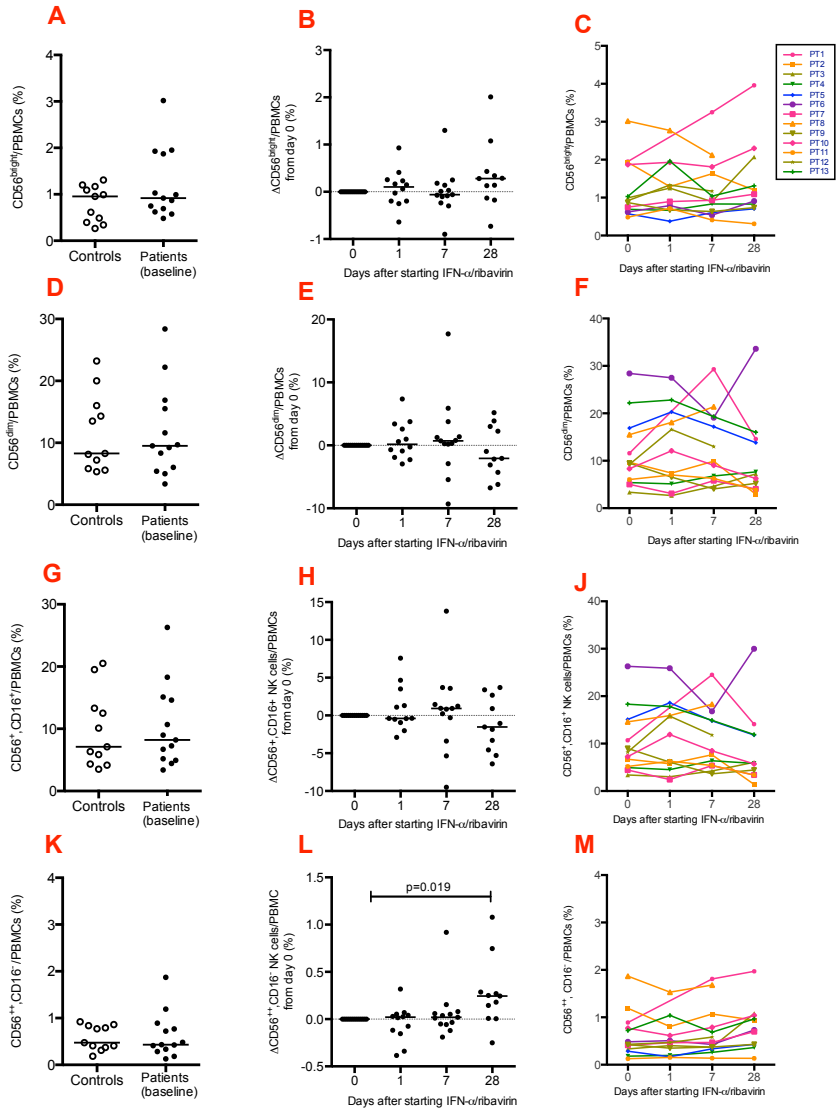
Figure 4-15; $CD56^{bright}$ NK cells as proportions of the NK cell population




Graph (A): Controls vs. patients at baseline. Graph (B): Values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Horizontal lines depict group medians.

Evaluating CD56$^{bright}$ and CD56$^{dim}$ subsets as independent PBMC subpopulations, there was no statistically significant change during therapy. However, CD56$^{++}$/CD16$^{-}$ NK cells rose significantly from 0.43% of PBMCs on day 0 to 0.73% on day 28 (median value, p = 0.02) (Figure 4-16L).

Figure 4-16; NK cell subpopulations as proportions of total PBMCs - longitudinal changes during HCV therapy in patients compared with controls




Graphs (A, B&C): CD56$^{bright}$ NK cells; (D, E&F): CD56$^{dim}$ NK cells; (G,H&J): CD56$^{+}$CD16$^{+}$ NK cells; and (K, L&M) CD56$^{++}$/CD16$^{-}$ NK cells. Left column: controls vs. patients at baseline. Middle column: Values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict group medians

### 4.6.6 DISCUSSION RELATING TO CHANGES IN CIRCULATING LYMPHOCYTE SUBSETS DURING THERAPY

The observation that lymphopenia occurs commonly during HCV therapy raises one obvious, but hitherto unanswered question – is lymphopenia caused by a decrease in one lymphocyte subset, or are all lymphocytes effected equally? Clearly, if a preferential decline in the periphery of one subset was driven by PEG-IFN/ribavirin therapy, this might impact on the immunogenicity of a concurrently administered therapeutic vaccine related to that cell subset's immune function.

A summary of significant results related to this subsection is presented in Table 4-4 below.

Table 4-4; Summary of significant baseline differences and longitudinal changes in lymphocyte subsets during HCV therapy

| | Healthy control | | Patient at baseline | Day 1 | | Day 28 | |
|---|---|---|---|---|---|---|---|
| **Cell subsets** | **% of respective subset** | | **% of respective subset** | **Δ% of respective subset from baseline** | | **Δ% of respective subset from baseline** | |
| $CD3^+$lymphocytes/PBMCs | | | 67.1 | | | ↑ 6.0 | p < 0.01 |
| $CD4^+$ | | | 68.4 | | | ↑ 2.1 | p = 0.04 |
| $CD8^+$ | | | 24.8 | ↓1.0 | p = 0.02 | | |
| $CD161^{bright}$ | 13.1 | p < 0.01 | 4.0 | ↓ 0.5 | p = 0.04 | | |
| Tregs | 2.5 | p = 0.02 | 4.2 | | | | |
| B cells/PBMCS | | | 9.2 | | | ↓ 3.3 | p = 0.02 |
| $NK^{bright}/NK^{dim}$ | 5.3 | p = 0.03 | 11.4 | | | ↑ 3.6 | p < 0.01 |

In the healthy control, day 1 and day 28 columns only values significantly different from HCV infected patients at baseline (shaded blue) are listed. Markers evaluated where no difference at baseline, or change during therapy was observed, are not included. p values for day 1 and day 28 are calculated using Wilcoxon signed rank test for matched pairs, and healthy control p values calculated using the Mann-Whitney U test. Values given are absolute % values of the respective cell subset.

Between day 0 and day 1 of PEG-IFN/ribavirin therapy, $CD8^+$ T cells decreased by 1.0% as a proportion of $CD3^+$ lymphocytes. After four weeks of therapy, an increase in $CD4^+$ T cells of 2.1% reached significance while a complementary decline in $CD8^+$ T cells did not. Both the decrease in B cells, and the complementary increase in $CD3^+$ lymphocytes (both as a subpopulations of PBMCs) over 28 days were significant.

It is not clear whether the minor changes in total $CD4^+$ and $CD8^+$ T cells and B cell populations early during therapy would directly impact on the efficacy of a concurrently administered vaccine.

The changes in $CD4^+$ and $CD8^+$ T cell populations observed in the THIRST study are in keeping with earlier work by Mohty et al. (Mohty et al. 2010). In their study, low-dose IFN-α given as an adjuvant during treatment for melanoma led to a decline in the total number of lymphocytes, with the greatest decrease in $CD8^+$ T cells (at 12 months, $CD8^+$ T cell: median relative decrease 75%).

### *Regulatory T cells*

The finding that compared with healthy individuals, patients with CHC have greater proportions of circulating Tregs has been previously described. Cabrera and colleagues demonstrated Tregs (defined as $CD4^+/CD25^+$ T cells) made up a higher proportion of PBMCs in 30 patients with CHC compared with 15 healthy controls (3.0% vs. 2.3%, p = 0.02) (Cabrera et al. 2004). Similarly, Ebinuma and colleagues found increased Treg cells (defined as $CD4^+/CD25^+$ high/$FoxP3^+$ T cells) in HCV infected patients (Ebinuma et al. 2008). In contrast however, Roe and colleagues found reduced proportions of Tregs in 15 HCV infected patients compared with healthy controls (Roe et al. 2009).

It has been suggested that because Tregs suppress proliferation of HCV-specific cytotoxic T cell responses, they play a central role in viral persistence in CHC (Rushbrook et al. 2005). The finding of an elevated number of circulating Tregs might help explain both chronicity, and the lower than expected number of circulating virus specific cytotoxic T cells found in patients with CHC compared with those seen in other chronic viral

infections. Indeed, elevated proportions of Treg cells have been found in liver biopsies of patients with CHC compared with other liver diseases (Rushbrook et al. 2005). However, others found no baseline difference in circulating Treg numbers (defined by $CD4^+/CD25^+/FoxP3^+$ cells) when comparing 62 CHC patients with 10 healthy controls (Burton et al. 2008).

Only one previous study has evaluated changes in circulating Tregs during therapy, (one time-point only after 8 weeks PEG-IFN/ribavirin therapy) (Burton et al. 2008). The authors do not present data on the changes in Tregs as proportions of circulating lymphocytes. However, they show no change in Treg phenotype as judged by FoxP3 expression (along with a number of other markers including CD62L and CD45RO) during therapy.

For regulatory T cells the picture is not clear – difficulties identifying subtle changes in number, particularly when their defining surface markers vary depending on cell activation, together with other factors such as the heterogeneity of CHC patients studied (eg. cirrhotic vs. non-cirrhotic) may significantly influence comparative results. Nevertheless, it is likely that Tregs play a role in limiting adaptive immune responses to HCV infection and therefore potential vaccine responses. The lack of a significant change in the proportion of circulating Tregs observed in the early stages of therapy suggests they play little role in determining early treatment response. To carry this finding forward we now plan to re-evaluate vaccine responders in the HCV003 trial in the context of their circulating Treg number – if there is any correlation between vaccine response and circulating Tregs, this might provide an explanation for the attenuated responses to vaccination observed in HCV infected patients.

### *NK cells*

In healthy individuals, NK cells typically comprise 5 - 20% of PBMCs but make up a substantially greater proportion of lymphocytes in the liver (30-50%) (Doherty et al. 1999). Unlike T and B cells, NK cells do not require priming. Instead, they possess a combination of activating receptors (for pathogens and cytokines), and inhibitory receptors (for host cells) that balance one another when the cell is in a resting state (McQueen & Parham 2002). When the balance tips in favor of activation, NK cells have a number of effector functions; i) they release Th1 cytokines (IFN-γ and TNF-α) to prime the adaptive immune response, and ii) they are directly cytotoxic, killing target cells via degranulation and surface expression of ligands such as TRAIL that activate death receptors.

In contrast to my work, several other studies have suggested that circulating total NK cell numbers are reduced in CHC (Meier et al. 2005; Golden-Mason et al. 2008). The clinical heterogeneity of patients evaluated across these different studies including factors such as; differing viral loads, diversity of HCV subtypes, variations in the duration of infection, and possibly the presence of other microbial infections, might provide some explanation for the diversity of these observations. Perhaps more importantly, there is evidence also to suggest NK function may be impaired in chronic HCV infection – for example; i) certain NK receptor genes predispose patients with acute HCV infection to develop chronic disease, (Khakoo et al. 2004) and, ii) HCV itself has been shown in-vitro to modulate NK cell activity by a number of means including direct induction of inhibitory NK cell ligands (Crotta et al. 2002; Tseng & Klimpel 2002).

In the THIRST study, the finding of an increased proportion of CD56$^{bright}$ NK cells within the total NK cell population before commencing treatment is in accordance with work by several others (Meier et al. 2005; Golden-Mason et al. 2008; Miyagi et al. 2011; Nattermann et al. 2006). For example, in 2005 Meier found a greater proportion of CD56$^{bright}$ NK cells (13.2% vs. 5.7%) in 36 HCV infected patients compared with 30 controls. I next demonstrated an expansion in this NK cell subset within 28 days of PEG-IFN/ribavirin therapy being commenced. Two previous studies demonstrated increased circulating CD5$^{bright}$ NK cells after 3 months PEG-IFN/ribavirin therapy (Lee et al. 2010b; Ahlenstiel et al. 2011) – one of these also evaluated days 7, 14 & 28 of therapy and reported an increase in both CD56$^{bright}$ and CD56$^{dim}$ NK cells (as percentages of lymphocytes) over the first two weeks, but no data on changes in their relative proportions is presented.

What are the implications of the finding that in HCV infected patients, CD56$^{bright}$ NK cells are relatively elevated, and this subpopulation increases during therapy? Unlike CD56$^{dim}$ NK cells, CD56$^{bright}$ NK do not express killer-cell immunoglobulin like receptors (KIRs), contain low levels of perforin, and are only weakly cytotoxic. Nevertheless, several studies have shown NK cell cytolytic activity in CHC infection is not impaired despite relative reductions in CD56$^{dim}$ NK cells (Okumura et al. 2005; Düesberg et al. 2001).

CD56$^{bright}$ NK cells express homing markers for secondary lymphoid organs and are important secretors of cytokines including IFN-γ, TNF-α and IL-10. The production of IFN-γ by NK cells is associated with both the induction of virus-specific T cell responses and liver injury in murine models of viral infection (Liu et al. 2000). Although failure of NK cell IFN-γ production has been reported in CHC infection (Meier et al. 2005; Ahlenstiel et al.

2010), the expansion of $CD56^{bright}$ NK cells in the liver could potentially result in IFN-γ mediated alterations in immune function and pathology.

Differences in the proportions of NK cell subpopulations may be explained by the unique expression of chemokine receptors by these cells. If chronic infection or therapy induces chemokine production, this may lead to differential sequestration of NK cell subpopulations depending on their chemokine receptor profile. Several researchers have provided preliminary support for this theory – though findings are inconsistent across studies, it is clear that subpopulations of NK cells express unique combinations of chemokine receptors. (Eberlein et al. 2010; Berahovich et al. 2006; Campbell et al. 2001). This will be further explored in Section 4.8 (page 177).

### *$CD161^{+}$ T cells*

CD161 is a C-type lectin, expressed on a significant subset of circulating HCV-specific T cells in patients with CHC and elevated proportions of such cells are found in tissue infiltrating populations and decrease in the periphery (Billerbeck et al. 2010). The finding of a close association with CD161 and chemokine receptor expression (CCR6 and CXCR6), originally suggested $CD161^{+}$ cells might play a unique role in targeting specific lymphocyte responses, particularly $CD8^{+}$ T cell responses to specific organs (eg. the liver) during CHC infection (Northfield et al. 2008). Further investigation has delineated 2 distinct subpopulations of $CD8^{+}$ T cells positive for CD161; $CD161^{bright}$ and $CD161^{dim}$ (see Figure 4-11), and these subpopulations display distinct phenotypical and homing characteristics.

$CD161^{bright}$ $CD8^{+}$ T cells, more recently defined as mucosal-associated invariant T (MAIT) cells, (Dusseaux et al. 2011) express patterns of molecules consistent with Th17

differentiation such as cytokines IL-17 and IL-22 and are typically CCR6$^{+}$/CXCR3 negative. These cells express the semi-invariant T-cell receptor and are selected by the MHC class Ib molecule, MR1 on hematopoietic cells. In contrast, CD161$^{dim}$ cells typically do not produce IL-17 upon stimulation and are CCR6$^{-}$/CXCR3$^{+}$.

Considering the contrasting chemokine receptor expression amongst CD161$^{+}$ CD8$^{+}$ subsets, just as it is for NK cell subsets, one possible explanation for the differential loss of CD8$^{+}$ CD161$^{bright}$ cells observed 1 day after administering PEG-IFN may relate to specific chemokine directed sequestration.

## 4.7 KEY QUESTION 2 – Does chronic HCV infection or PEG-IFN/ribavirin therapy change the cytotoxicity of circulating lymphocytes?

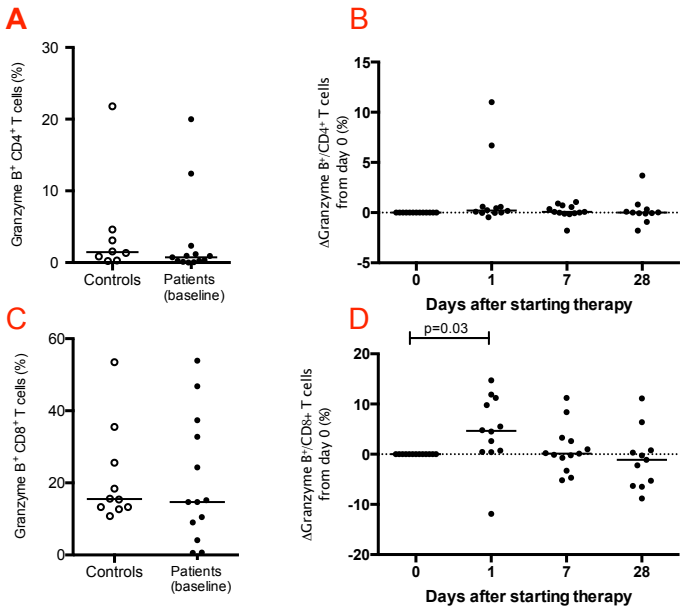
One major pathway for inducing apoptosis of virus-infected cells, is perforin mediated delivery of granzymes into target cells by $CD8^+$ T cells (Kurschus & Jenne 2010). Perforin and Granzyme B expression were therefore chosen for evaluation as markers of lymphocyte cytotoxic capacity in the THIRST trial.

### 4.7.1 PERFORIN AND GRANZYME B EXPRESSION

At baseline, 8.2% of PBMCs and 1.4% of $CD3^+$ cells were perforin positive in the CHC population, with similar levels observed in the healthy control group (10.7% and 2% respectively, median values) (Appendix 11). Similarly, there were no baseline differences in granzyme B expression between HCV infected patients and controls.

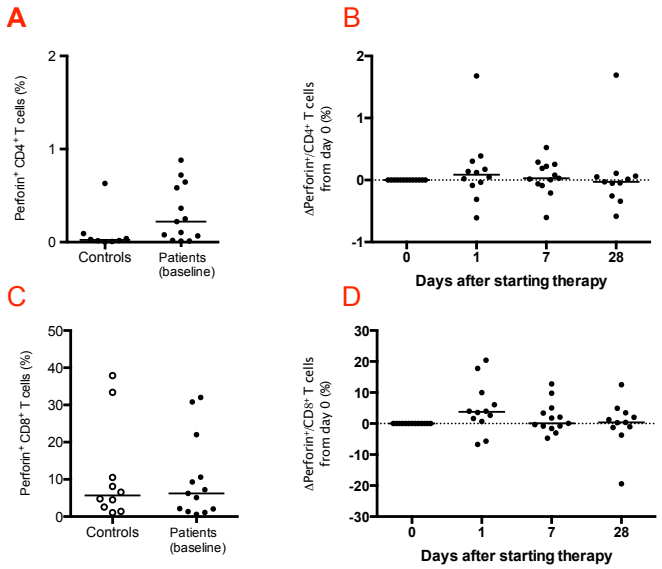
One day after commencing PEG-IFN/ribavirin therapy, expression of granzyme B by $CD8^+$ T cells rose by a median of 4.7% ($p = 0.03$) (Figure 4-17). A similar change in perforin expression by $CD4^+$ cells was not statistically significant ($p = 0.08$) (Figure 4-19). Over the following 4 weeks therapy these levels returned to baseline. There were no other significant changes in either perforin or granzyme B expression during therapy. It should be noted that inter-individual expression of perforin and granzyme B was highly variable (0.6-32% and 0.6-53.9% of $CD4^+$ and $CD8^+$ T cells respectively). There was however, relatively minor variation only across sequential time-points for individual patients.

Figure 4-17; Granzyme B expression by T cells – longitudinal changes during HCV therapy in patients compared with controls




Graphs (A&B): $CD4^+$ T cells. Graphs (C&D): $CD8^+$ T cells. Horizontal lines depict group medians. Right column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline

Figure 4-18; Perforin expression by T cells – longitudinal changes during HCV therapy in patients compared with controls




Graphs (A&B): $CD4^+$ T cells. Graphs (C&D): $CD8^+$ T cells. Horizontal lines depict group medians. Right column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline

### 4.7.2 DISCUSSION RELATING TO CYTOTOXIC CAPACITY

There was no significant difference identified in the cytotoxic capacity of circulating lymphocytes comparing HCV infected patients and healthy controls as judged by perforin and granzyme B expression. During PEG-IFN/ribavirin therapy, the only significant change identified was a 4.7% increase in granzyme B expression by $CD8^+$ T cells 1 day after commencing therapy.

Unlike the THIRST study, previous work has suggested perforin expression by $CD3^+$ lymphocytes is globally reduced in the setting of chronic HCV infection and increases with therapy. In 2002, Par and colleagues evaluated $CD3^+$ and $CD3^-$ lymphocyte perforin levels in 9 patients undergoing non-pegylated IFN-α therapy (Pár et al. 2002). At baseline, 16.9% of total lymphocytes were perforin positive compared with 29.9% of lymphocytes in healthy controls. After 1 month of therapy they found perforin positive lymphocytes increased from ~17 to ~28%, and this was sustained in those patients who achieved an SVR.

It should also be noted that, like my own study, the perforin antibody clone (δG9) used in the study by Par and colleagues allows detection of pre-formed perforin only. There is a more recent clone (B-D48) that allows detection of both granule associated and granule independent perforin that is up-regulated following antigen stimulation (Hersperger et al. 2008) – this may more accurately reflect the true cytotoxic capacity of lymphocytes. Using this clone, recent work evaluating HCV specific CD8+ T cell responses has demonstrated that the HCV specific T cell population expresses low levels of perforin and has an impaired ability to upregulate perforin when activated (Jo et al. 2012).

The use of concomitant ribavirin in the THIRST trial may explain partly the failure to observe an increase in perforin levels during therapy similar to that of the Par study. A recent study by Ogbomo demonstrated impaired production of perforin by NK cells following ribavirin exposure through down-regulation of IL-15 receptor expression (Ogbomo et al. 2010). It is not unreasonable to expect that a similar effect might be observed on $CD8^+$ T cells, given approximately 40% of these cells express the IL-15 receptor (Schneider et al. 2011). Nevertheless, the consistent levels of perforin and granzyme B over the course of early treatment gives some reassurance that the overall cytotoxic capacity of lymphocytes is not impaired by PEG-IFN/ribavirin therapy and this should not change the cytotoxic effector capacity of vaccine induced T cells.

## 4.8 KEY QUESTION 3 – Does HCV infection or PEG-IFN/ribavirin therapy change the homing phenotype of PBMCs?

CXCR3 and CCR6 are chemokine receptors expressed on a range of lymphocyte subpopulations and are important in directing homing of lymphocytes to inflamed livers. These receptors were therefore chosen for evaluation in the THIRST trial.

Since CXCR3 and CCR6 staining does not typically yield distinct positive and negative cell populations by flow cytometry, $CD8^+$ $CD161^{bright}$ cells, that are typically CXCR3 negative/CCR6 positive (Billerbeck et al. 2010) were used to define FACS gates for the CXCR3/CCR6 populations, as demonstrated in Figure 4-19. Once established, gating position was kept constant for each individual across all time-points.

Figure 4-19; FACs plot demonstrating gating strategy used to define $CXCR3^+$ cells

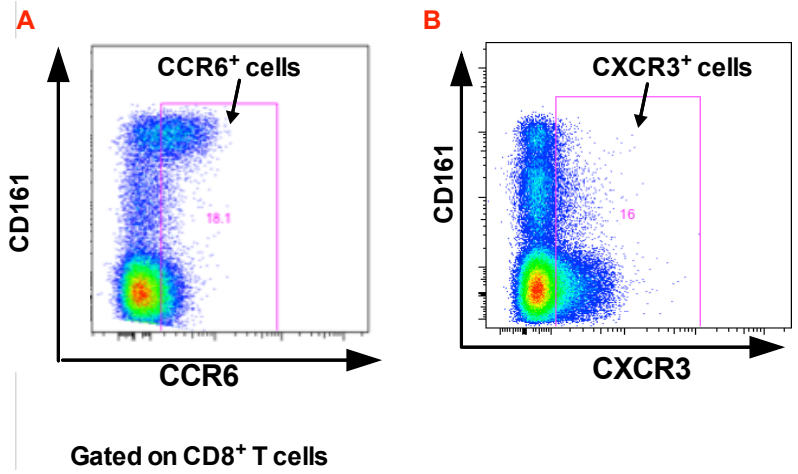
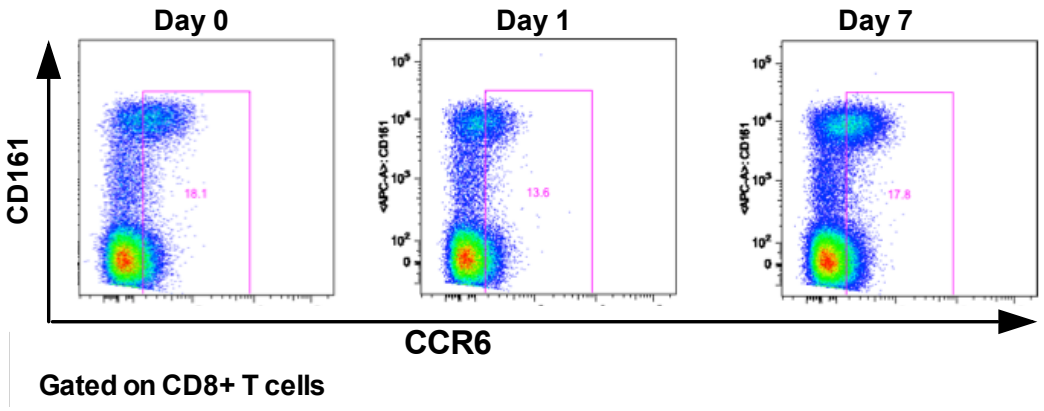



Figure (A): CCR6 expression on $CD161^+$ cells. Figure (B): CXCR3 expression on $CD161^+$ cells Representative FACS plots from a healthy volunteer gated on single cells/PBMCs/live cells/$CD3^+$/$CD8^+$ cells. $CD161^{bright}$ cells (predominantly positive for CCR6 and negative for CXCR3) were used to establish gates for these chemokine receptors as demonstrated.

### 4.8.1 CCR6 EXPRESSION

Example FACS plots demonstrating the longitudinal change during therapy in CCR6 expression by CD161$^{bright}$ cells for an individual patient is provided in Figure 4-20.

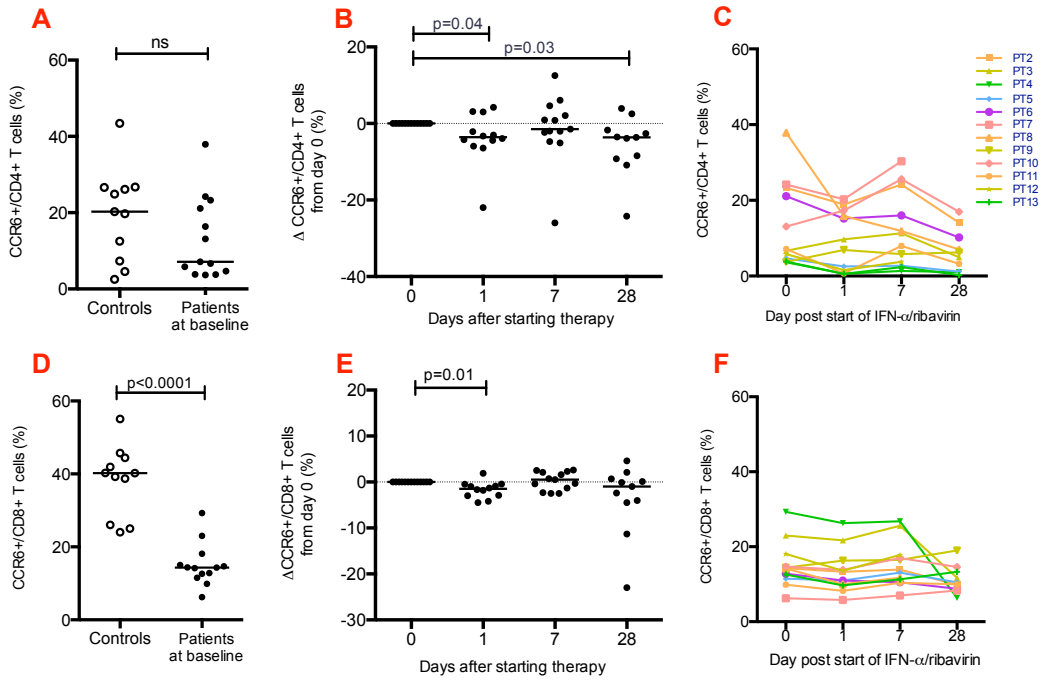
Figure 4-20; FACS plot demonstrating CCR6 gating strategy together with longitudinal change in its expression during therapy




The purple rectangle gates on CCR6$^{+}$ cells. These plots have first been gated on CD8$^{+}$ T cells and are all from the same individual patient

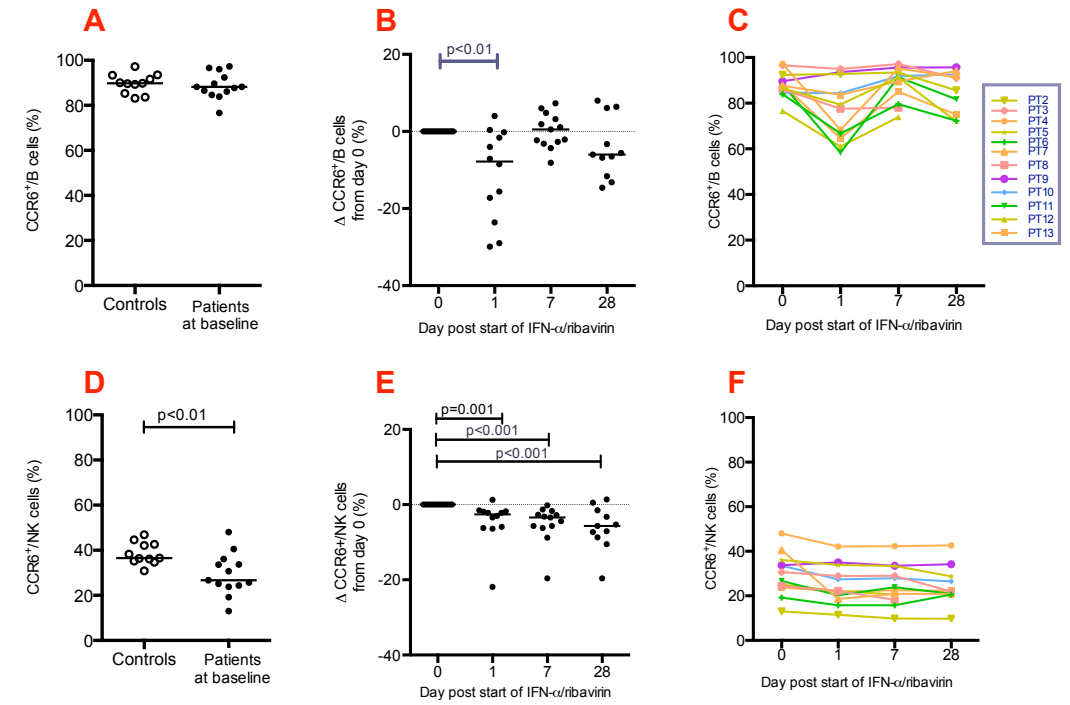
At baseline, CD8$^{+}$ T cells and NK cells from patients express significantly less CCR6 than healthy controls (14.3% vs. 40.2%, $p < 0.0001$ and 26.7% vs. 36.5%, $p < 0.01$ respectively) (Figure 4-21 and Figure 4-22). Between day 0 and day 1 of therapy, CCR6 expression decreased on all lymphocyte subsets (CD8$^{+}$: -1.7%, CD4$^{+}$: -3.6%, B cells -7.8% and NK cells -2.6%, $p<0.05$ for all, median changes). After 28 days therapy, the proportion of CD4$^{+}$ T cells and NK cells expressing CCR6 remained lower than baseline (-3.6%, $p = 0.03$ and -0.7% respectively, $p < 0.001$).

Figure 4-21; CCR6 expression by T cells - longitudinal changes during HCV therapy in patients compared with controls




Top row (A, B & C) represents $CD4^+$ T cells. Bottom row (D, E & F) represents $CD8^+$ T cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point. ns = not significant

Figure 4-22; CCR6 expression by B cells and NK cells; longitudinal changes during HCV therapy in patients compared with controls

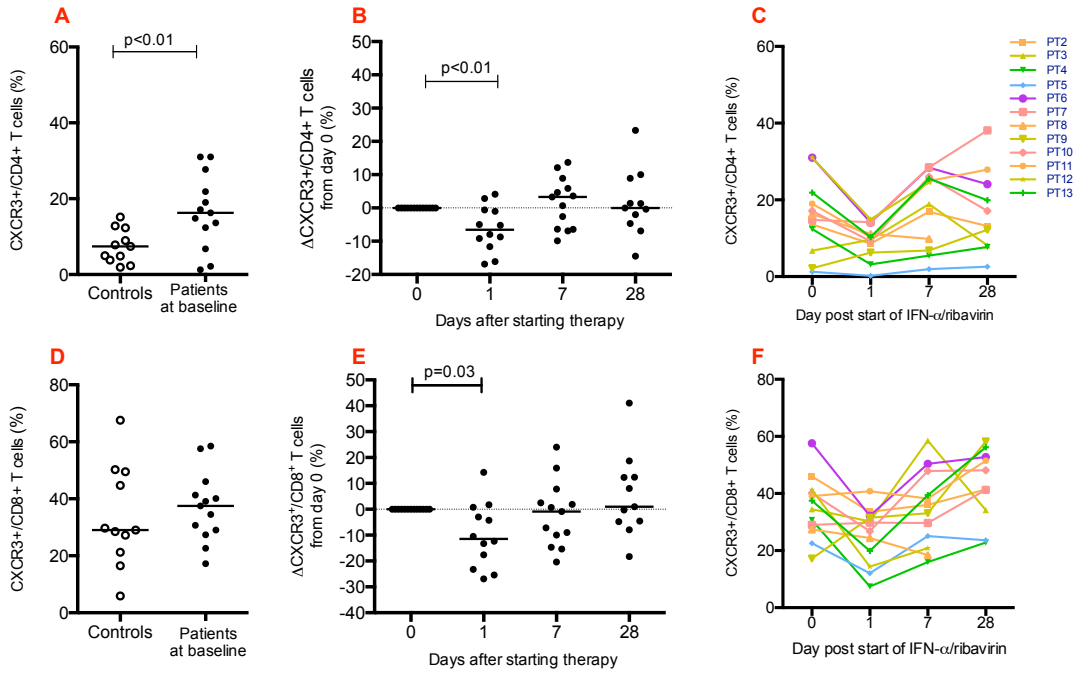



Top row: B cells. Bottom row: NK cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

## 4.8.2 CXCR3 EXPRESSION

At baseline, $CD4^+$ T cells from HCV infected patients expressed significantly more CXCR3 than those of healthy controls (16.3% vs. 7.4%, median values, $p < 0.01$), as did B cells (53.2% vs. 30.6%, $p < 0.01$). One day after commencing therapy, CXCR3 expression on $CD4^+$, $CD8^+$ T cells (Figure 4-23) and B cells (Figure 4-24) decreased strikingly by medians of 40.0%, 24.5% and 39.8% respectively (CD4: 16.5% → 9.9%, $p < 0.01$, $CD8^+$: 37.5% → 28.3%, $p = 0.03$ and B cells: 53.2% → 32.0%, $p < 0.01$, all are absolute changes). This change was also confirmed using median fluorescence intensity of CXCR3 expression (Appendix 12).

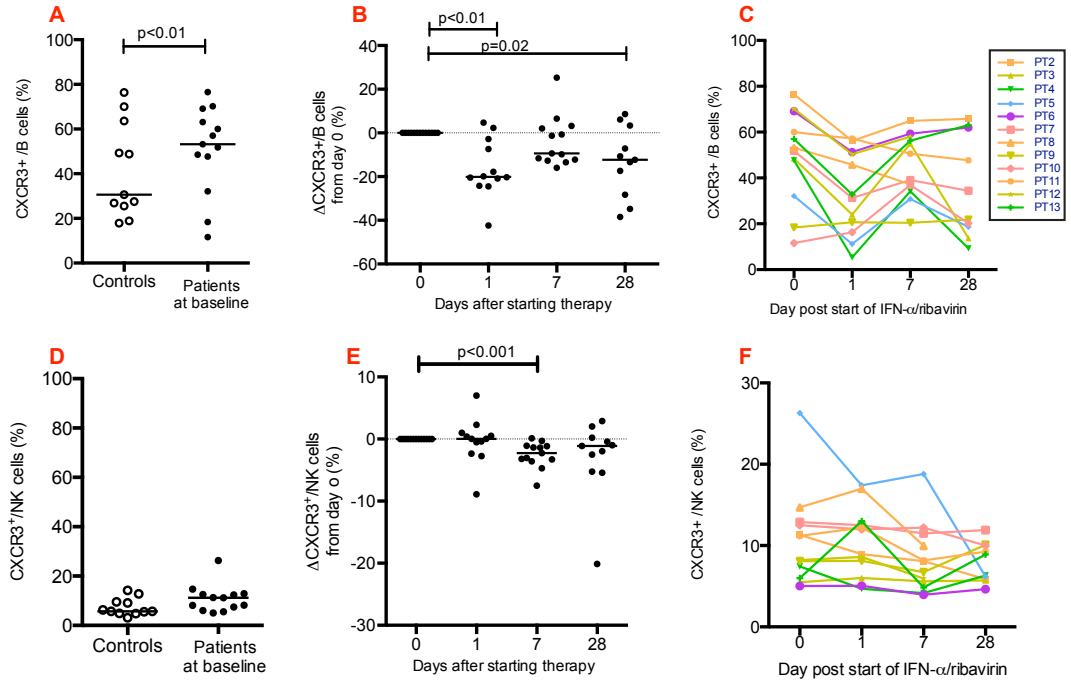
Figure 4-23; CXCR3 expression by T cells - longitudinal changes during HCV therapy in patients compared with controls




Top row: $CD4^+$ T cells. Bottom row: $CD8^+$ T cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

Unlike CXCR3 expression by T cells (which had returned to pretreatment levels by day 7) CXCR3 expression by B cells fell by a median of 12.3% at day 28 (p = 0.02). A delayed decline in CXCR3+ NK cells was also observed at day 7 (-1.0%, median change, p < 0.001) (Figure 4-24-D).

Figure 4-24; CXCR3 expression by B cells and NK cells - longitudinal changes during HCV therapy in patients compared with controls

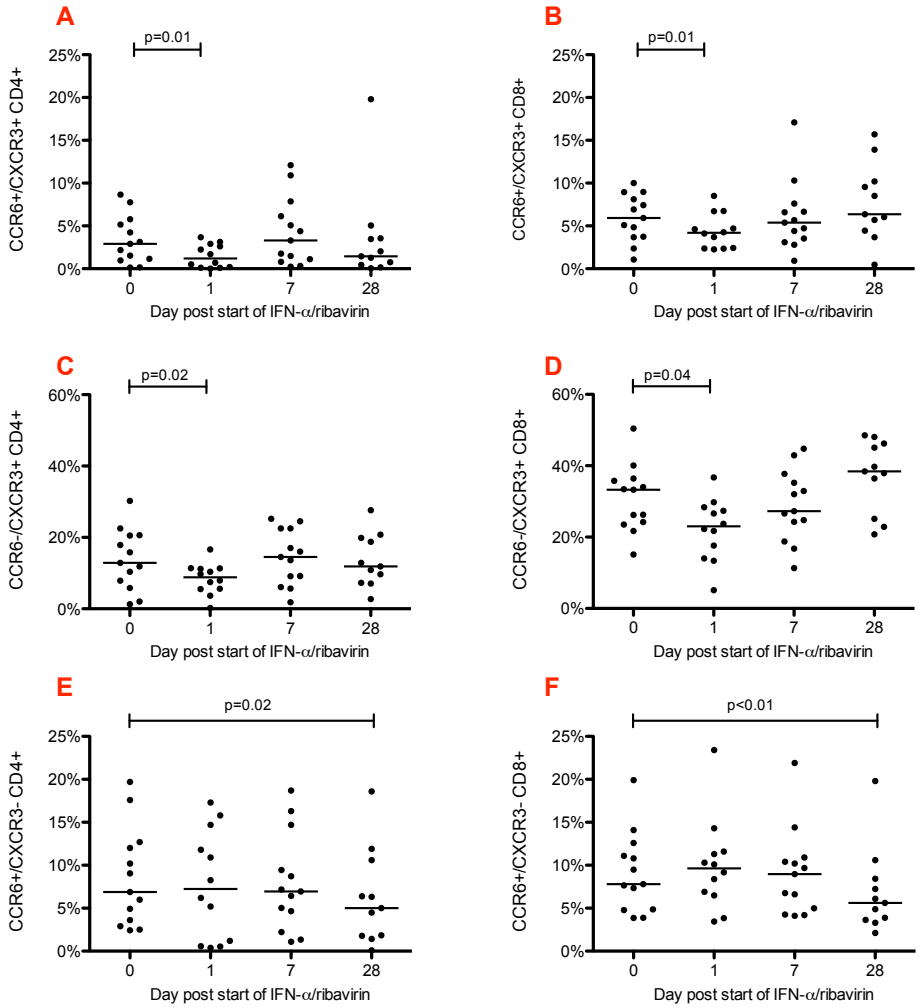



Top row: B cells. Bottom row: NK cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

### 4.8.3 DIFFERENTIAL EXPRESSION OF CCR6 AND CXCR3 BY T CELLS

Given the shared function of CCR6 and CXCR3 in driving hepatic homing of lymphocytes, their co-expression was evaluated on $CD4^+$ and $CD8^+$ T cells (Figure 4-25). At baseline, in HCV infected patients, 2.9% of $CD4^+$ T cells and 5.9% of $CD8^+$ T cells co-expressed CCR6 and CXCR3.

Figure 4-25; Differential co-expression of CCR6 and CXCR3 by $CD4^+$ and $CD8^+$ T cells during HCV therapy

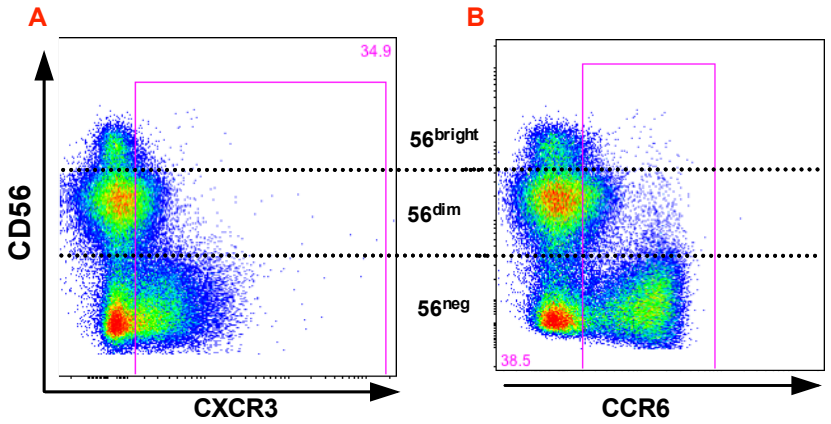



The top row (A&B) of graphs represent cells co-expressing CCR6 and CXCR3. The middle row (C&D) represents $CXCR3^+/CCR6^-$ cells and the bottom row (E&F) represents $CCR6^+/CXCR3^-$ cell populations. Horizontal lines depict medians at each time-point.

Interestingly, 1 day after beginning therapy, only $CD4^+$ and $CD8^+$ $CCR6^+$ cells that co-expressed CXCR3 decreased ($p = 0.01$) while $CCR6^+/CXCR3^-$ cells remained static – this suggests that CXCR3 is the driving factor behind the day 1 decrease in $CCR6^+/CXCR3^+$ T cells. It should also be noted that the decrease in $CCR6^+$ cells at day 28 was isolated to the $CCR6^+/CXCR3^-$ T cell population – indicating in a similar manner that, later during therapy, CCR6 dependent lymphocyte redistribution may play a more significant role in the changes observed.

Based on the observation that $CD56^{bright}$ cells decreased more significantly during therapy than $CD56^{dim}$ cells (see Section 4.6.5, page 163), differential expression of CCR6 and CXCR3 was also evaluated for these NK cell subsets. I found that greater proportions of $CD56^{dim}$ NK cells expressed CXCR3 and CCR6 than their $CD56^{bright}$ counterparts (17.1% vs. 7.4% for CXCR3, median baseline percentage, $p < 0.01$) (Figure 4-26).

Figure 4-26; FACS plots demonstrating CXCR3 and CCR6 expression by NK cell subpopulations




CXCR3 expression (A) and CCR6 (B) expression by CD56 bright, dim and negative cell populations. These plots have been first gated on $CD3^-$ PBMCs

## 4.8.4 DISCUSSION RELATING TO LYMPHOCYTE HOMING

A summary of significant results related to this subsection is presented in Table 4-5 below.

Table 4-5; Chemokine receptor expression by circulating lymphocytes - significant baseline differences and longitudinal changes during therapy in HCV infected patients

| | | Healthy control | | Patient at baseline | Day 1 | | Day 28 | |
|---|---|---|---|---|---|---|---|---|
| **Cell subsets** | | **% of respective subset** | | **% of respective subset** | **Δ% of respective subset from baseline** | | **Δ% of respective subset from baseline** | |
| **Homing marker** | | | | | | | | |
| CXCR3 | $CD4^+$ | 7.4 | p < 0.01 | 16.3 | ↓ 3.6 | p < 0.01 | | |
| | $CD8^+$ | | | 37.5 | ↓ 11.5 | p = 0.03 | | |
| | B cells | 30.6 | p < 0.01 | 53.2 | ↓ 20.1 | p < 0.01 | ↓12.3 | p = 0.02 |
| | NK cells | | | 11.2 | ↓ 2.3 (day 7) | p < 0.01 | | |
| CCR6 | $CD4^+$ | | | 7.1 | ↓ 1.7 | p = 0.04 | ↓ 3.6 | p = 0.03 |
| | $CD8^+$ | 40.2 | p< 0.0001 | 14.3 | ↓ 3.6 | p = 0.01 | | |
| | NK cells | 36.5 | p < 0.01 | 26.7 | ↓ 7.8 | p= 0.001 | ↓ 5.7 | p < 0.001 |

In the healthy control, day 1 and day 28 columns only values significantly different from HCV infected patients at baseline (shaded blue) are listed. Markers evaluated where no difference at baseline, or change during therapy was observed, are not included. p values for day 1 and day 28 are calculated using Wilcoxon signed rank test for matched pairs, and healthy control p values calculated using the Mann-Whitney U test. Values given are absolute % values of the respective cell subset.

CCR6 is a chemokine receptor expressed on lymphocytes and antigen presenting cells. It has a single chemokine ligand, CCL20 (or MIP-3-α) (Baba et al. 1997) that that is expressed in lung, liver, appendix, tonsillar crypts and epithelium (Hieshima et al. 1997; Power et al. 1997; Dieu et al. 1998). Plasma CCL20 levels have been shown to be elevated in CHC, correlate with ALT and interface hepatitis, and increase during therapy in patients who achieve an SVR (Yamauchi et al. 2002).

It is likely that the lower number of $CCR6^+$ $CD8^+$ T cells in HCV infected patients is explained, at least in part, by the reduced proportion of circulating $CD161^{bright}$ $CD8^+$ T cells because these cells express high levels of CCR6 (see Section 4.6.4, page 161) (Billerbeck et al. 2010). Theoretically, elevated CCL20 in the setting of chronic HCV infection might explain lower baseline levels of circulating $CCR6^+$ cells through target organ sequestration (Yamauchi et al. 2002).

Like CCR6, CXCR3 is a chemokine receptor widely expressed on immune cells including neutrophils and lymphocytes ($CD4^+$, $CD8^+$, NK cells and NK T cells). It is partly responsible for hepatic homing of these cells during inflammation and $CXCR3^+$ lymphocytes are enriched in the liver of patients with CHC infection (J. Wang et al. 2004).

This is the second study to demonstrate increased expression of CXCR3 by circulating $CD4^+$ T cells in HCV infected patients. A 2009 study by Roe and colleagues similarly found elevated proportions of circulating CXCR3+ CD4+ T cells in 15 HCV infected patients compared with healthy controls (Roe et al. 2009). In an earlier study, circulating B cells expressing CXCR3 were also shown to be increased in HCV infected patients (Butera et al. 2005). Considering plasma CXCL10 (a chemokine that binds CXCR3) is elevated in patients with CHC infection, an elevation in circulating CXCR3 expressing lymphocytes appears paradoxical because these cells should theoretically be sequestered in target organs. This may however, be explained by the recent finding that a truncated, antagonistic form of CXCL10 predominates in circulation in patients with chronic HCV infection (Casrouge et al. 2011) .

Early during therapy, one appealing explanation for the observed decrease in circulating $CXCR3^+$ and $CCR6^+$ cells is that PEG-IFN/ribavirin induces chemokine mediated hepatic recruitment of circulating lymphocytes. Combined with our previous observation of a direct correlation between lymphopenia and viral clearance, (Humphreys et al. 2012) this would indirectly suggest a role for liver recruited T cells in viral clearance during HCV therapy.

An increase in CXCR3-binding chemokines during therapy might also explain the early proportional rise in circulating $CD56^{bright}$ NK cells because this subset is relatively low in CXCR3 (compared with $CD5^{dim}$ NK cells) and would therefore remain in circulation. We must however, be careful in drawing too strong a link between these combined findings - conflicting data on CXCR3 receptor expression amongst NK cell subsets has been published (Berahovich et al. 2006; Campbell et al. 2001), samples numbers are small, and the differences I found in circulating subset proportions though significant, are minor.

Interestingly, when co-expression of CCR6 and CXCR3 by T cells was evaluated, it became clear that CXCR3 was the likely mediator behind the observed change on day 1 of therapy – only $CCR6^{+}$ T cells that co-expressed CXCR3 decreased significantly between day 0 and day 1. The fact that the substantial decrease in CXCR3 expression was only transient might be explained by a similarly transient increase in plasma CXCR3-binding chemokines. If this is the case, it might also explain why this phenomenon has not previously been described during HCV therapy.

These novel findings are particularly interesting as they provide evidence suggesting that HCV therapy induced lymphopenia may be driven in part by CXCR3 mediated lymphocyte sequestration. To further investigate this hypothesis a detailed analysis of CXCR3 and its related chemokines, both in the setting of chronic HCV infection, and during therapy will form the basis of Chapter 5 of this thesis.

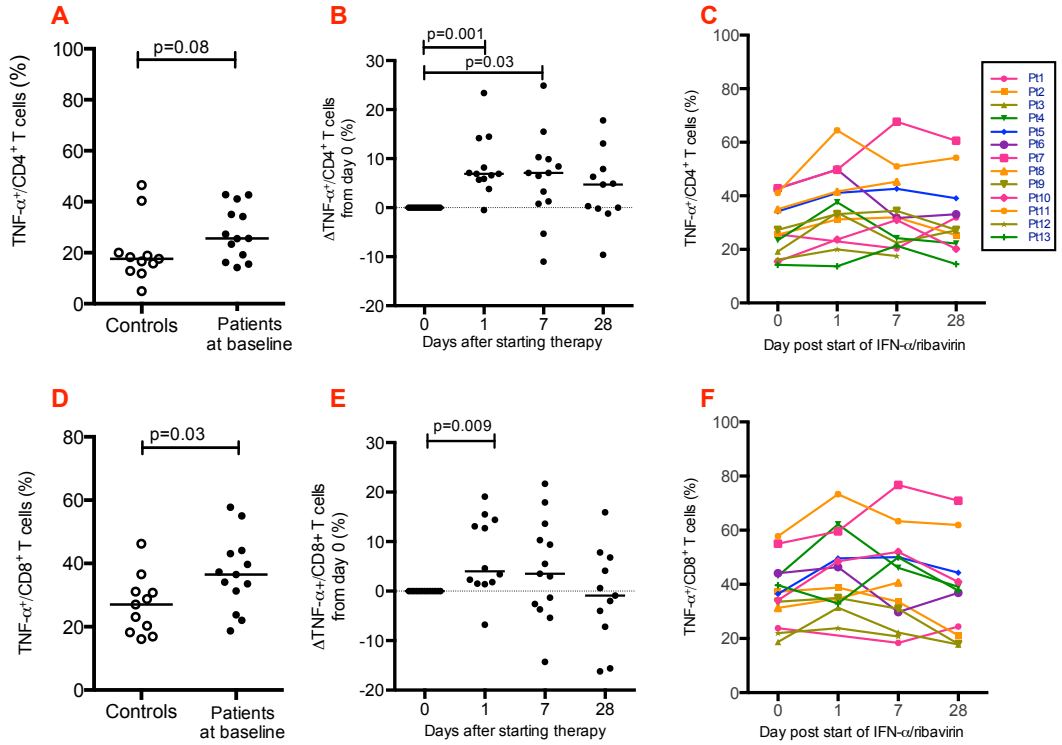
## 4.9 KEY QUESTION 4 - Does HCV infection or PEG-IFN/ribavirin therapy change cytokine production by PBMCs?

The cytokine profile of PBMCs was assessed after stimulation ex-vivo with PMA/ionomycin for 6 hours (see Methods 2.8.3, page 82). Specifically, the cytokines I assessed were; TNF-α, IL-17, IFN-γ and IL-2.

### 4.9.1 TNF-α

$CD8^+$ T cells from patients with CHC are more capable of producing TNF-α than healthy controls (36.5% vs. 27.0% of $CD8^+$ T cells, median values, p = 0.03) (Figure 4-27). A similar but non-significant trend was also observed for $CD4^+$ T cells (25.6% vs. 17.6%, p = 0.08). One day after commencing therapy, higher proportions of $CD4^+$ and $CD8^+$ T cells produce TNF-α (increase by 6.9% and 4% respectively), although this remains significant at day 7 for $CD4^+$ T cells only (+7.1%, p = 0.03). By day 28, TNF-α production by both cell populations had returned to baseline.

Figure 4-27; TNF-α production – baseline and longitudinal changes during HCV therapy in HCV infected patients compared with controls

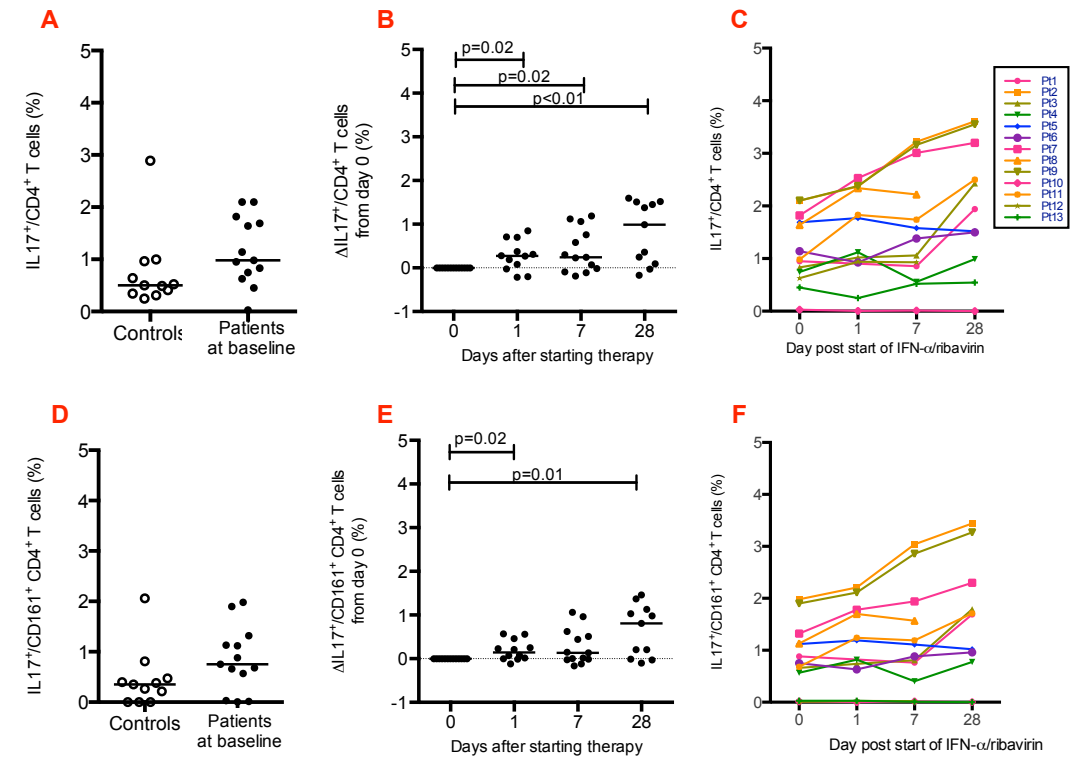



Top row: $CD4^+$ T cells; Bottom row: $CD8^+$ T cells. Cells were stimulated for 6 hours with PMA/ionomycin. At each time-point cytokine production is normalised against unstimulated cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

### 4.9.2 IL-17

There was no difference in IL-17 production by $CD4^+$ T cells between HCV infected patients and healthy controls. However, a progressive increase in IL-17 production was observed during therapy (day 28; +1.0%, median change, $p < 0.01$) (Figure 4-28). Interestingly, there was a suggestion those patients who achieved an RVR, were also those with the greatest increase in IL-17 production (Figure 4-29).

Figure 4-28; IL-17 production - baseline and longitudinal changes in during HCV therapy in patients compared with healthy controls




Top row: Total $CD4^+$ T cells. Bottom row: $CD4^+$ $CD161^+$ T cells. Cells were stimulated for 6 hours with PMA/ionomycin. At each time-point cytokine production is normalised against unstimulated cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

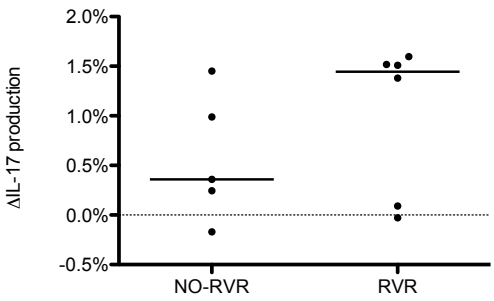


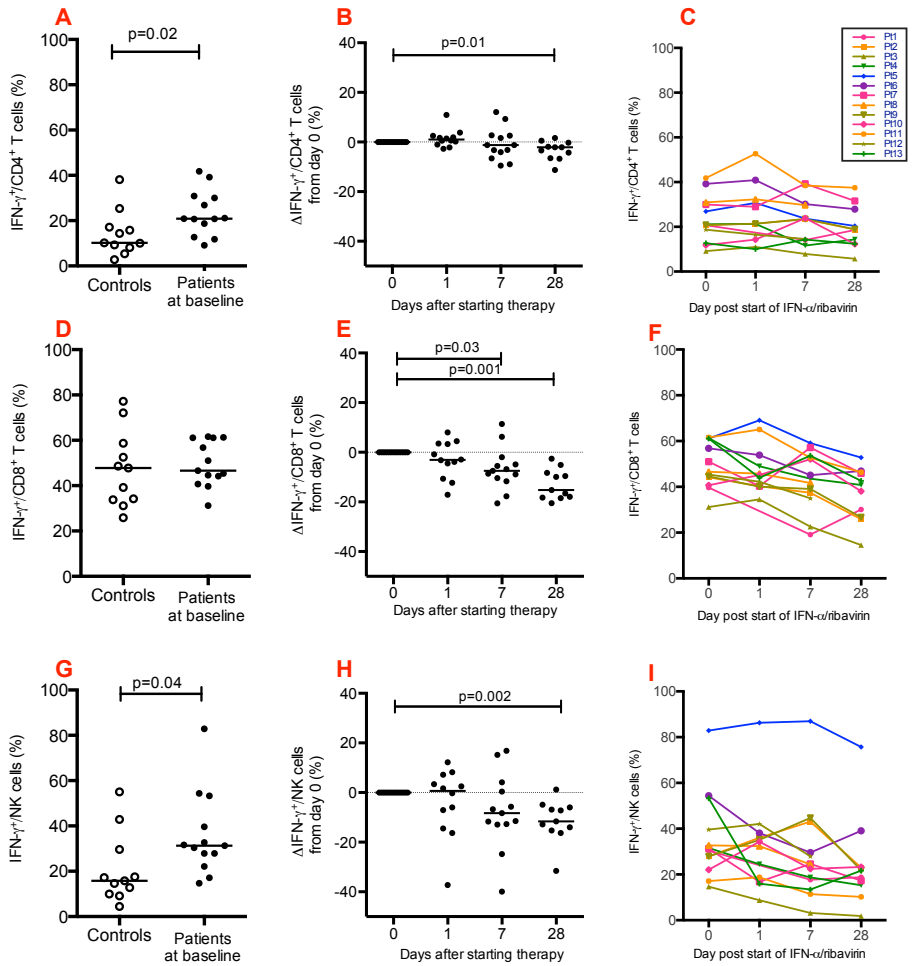

Figure 4-29; ΔIL-17 production by CD4+ T cells after 4 weeks therapy (day 28 – day 0)

Patients who achieved an RVR compared with those who did not. Horizontal lines depict median values.

### 4.9.3 IFN-γ

CD4+ T cells and NK cells (but not CD8+ T cells) from HCV infected patients are more able to produce IFN-γ than healthy controls (CD4: 20.9% vs. 10.2%, p = 0.02 and NK: 31.3% vs. 15.8%, p = 0.04, median values). During therapy, IFN-γ production by CD4+, CD8+ T cells and NK cells decreased by medians of 2.1%, 15.2%, and 11.6% respectively (Figure 4-30).

Figure 4-30; Longitudinal changes in IFN-γ production by lymphocytes during therapy in HCV infected patients compared with healthy controls

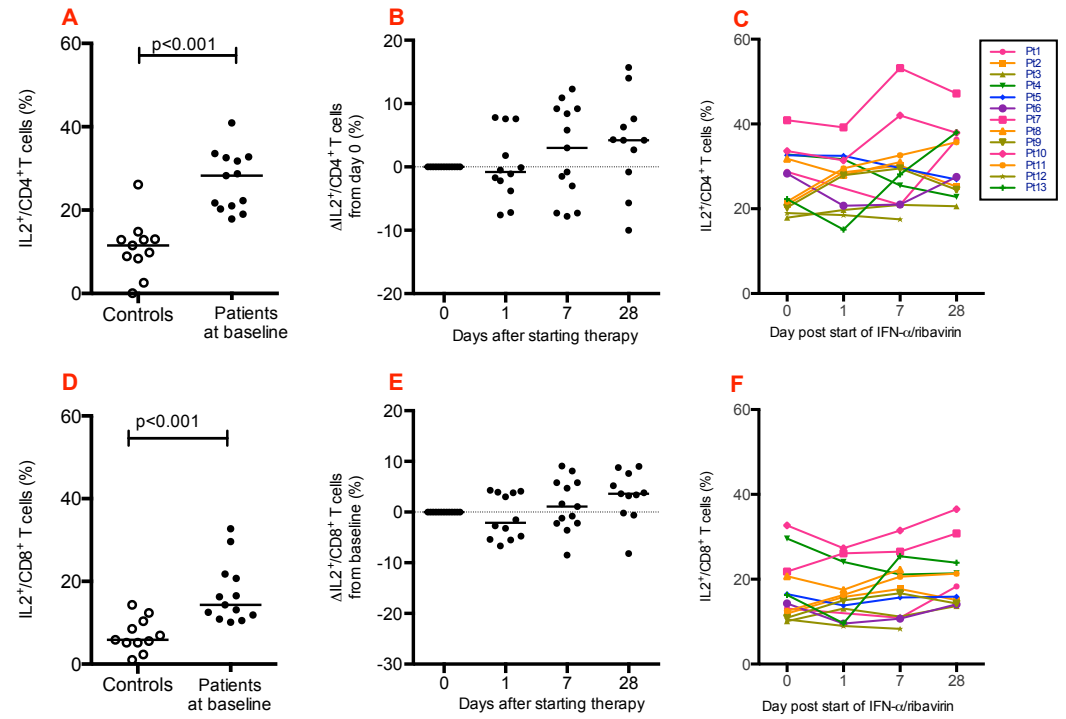



Top row: CD4+ T cells. Middle row: CD8+ T cells. Bottom row: NK Cells. Cells were stimulated for 6 hours with PMA/ionomycin. At each time-point cytokine production is normalised against unstimulated cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

### 4.9.4 IL-2

At baseline, greater proportions of $CD4^+$ and $CD8^+$ T cells from patients produce IL-2 than healthy controls (CD4: 27.0 vs. 11.0%, $p < 0.001$ and CD8: 17.0 vs. 7.1%, $p < 0.001$, median values) (Figure 4-31). During therapy, there was no statistically significant change in IL-2 production.

Figure 4-31; Longitudinal changes in IL-2 production by lymphocytes during therapy in HCV infected patients compared with healthy controls




Top row: $CD4^+$ T cells. Bottom row: $CD8^+$ T cells. Cells were stimulated for 6 hours with PMA/ionomycin. At each time-point cytokine production is normalised against unstimulated cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

## 4.9.5 DISCUSSION RELATING TO CHANGES IN LYMPHOCYTE CYTOKINE PROFILE

A summary of significant results related to this subsection is presented in Table 4-6 below.

Table 4-6; Significant baseline differences and longitudinal changes in circulating lymphocyte cytokine profiles during HCV therapy

| | | Healthy control | | Patient at baseline | Day 1 | | Day 28 | |
|---|---|---|---|---|---|---|---|---|
| **Cell subsets** | | **% of respective subset** | | **% of respective subset** | **Δ% of respective subset from baseline** | | **Δ% of respective subset from baseline** | |
| **Functional changes** | | | | | | | | |
| IFN-γ | $CD4^+$ | 14.2 | p = 0.02 | 23.4 | | | ↓ 2.1 | p = 0.01 |
| | $CD8^+$ | | | 46.6 | | | ↓ 15.2 | p = 0.001 |
| | NK cells | 20.8 | p = 0.04 | 35.8 | | | ↓ 11.6 | p = 0.002 |
| IL-2 | $CD4^+$ | 11.0 | p < 0.001 | 27.0 | | | | |
| | $CD8^+$ | 7.1 | p < 0.001 | 17.0 | | | | |
| IL-17 | $CD4^+$ | | | 0.50 | ↑ 1.0 | p = 0.02 | ↑ 1.0 | p < 0.01 |
| TNF-α | $CD4^+$ | | | 25.6 | ↑ 6.9 | p = 0.001 | | |
| | $CD8^+$ | 27.0 | p = 0.03 | 36.5 | ↑ 4.0 | p < 0.01 | | |

In the healthy control, day 1 and day 28 columns only values significantly different from HCV infected patients at baseline (shaded blue) are listed. Markers evaluated where no difference at baseline, or change during therapy was observed, are not included. P values for day 1 and day 28 are calculated using Wilcoxon signed rank test for matched pairs, and healthy control p values calculated using the Mann-Whitney U test. Values given are absolute % values of the respective cell subset.

Lymphocytes from HCV infected patients have a higher functional reserve for the production of the pro-inflammatory cytokines, TNF-α, IFN-γ and IL-2. Interestingly, during the first 28 days of PEG-IFN/ribavirin therapy, this reserve declines for IFN-γ, while it increases for Il-17 and TNF-α.

The reasons for the differences, both in the baseline capacity to produce cytokines and changes observed during therapy in patients with CHC, are likely to be complex and multifactorial. HCV infection itself may impair cytokine production indirectly by promoting lymphocyte senescence, but also directly through mechanisms such as viral protein mediated inhibition of cytokine production (Neumann-Haefelin 2005). During therapy therefore, changes in lymphocyte cytokine production might simply reflect

reductions in circulating virus levels. However, it is also likely that exogenously administered PEG-IFN and ribavirin contribute to the changes observed.

The impact of these combined functional changes on the successful application of a therapeutic vaccine are difficult to interpret. IL-2 is important for the induction of naïve T cells and the generation of an appropriate effector memory T cell response – it is therefore reassuring that T cell capacity to produce this cytokine during therapy is unchanged. Meanwhile, paradoxical changes in the pro-inflammatory cytokines TNF-α and IFN-γ are likely to exert global effects on T cell induction that are difficult to interpret in isolation. Nevertheless, a summary of the functional role of these and the specific mechanisms that might explain the differences observed in the THIRST study is as follows:

### *Tumor Necrosis Factor-α*

Tumor Necrosis Factor-α (TNF-α) is an inflammatory cytokine that is mainly produced by macrophages. A number of other cell types including lymphocytes also produce this cytokine, initially as a transmembrane protein that is then cleaved by TNF-α converting enzyme (TACE) and released in a soluble form. TNF-α has 2 receptors; i) TNF-R1, which is widely expressed and binds both soluble and membrane bound TNF-α, while ii) TNF-R2, which is found on lymphocytes, can only bind membrane associated TNF-α. Following ligand binding 3 complex pathways involved with cell proliferation, differentiation and apoptosis are activated; NF-κB, MAPK and death signaling (Wajant et al. 2003). Ultimately, TNF-α promotes a complex inflammatory response, the features of which depend on a range of concurrent factors such as the surrounding cell types and cytokine milieu.

The finding of an increased capacity for $CD8^+$ T cells to produce TNF-α in HCV infected patients compared with healthy controls is in keeping with work by Itoh and Zylerberg

(Itoh et al. 1999; Zylberberg et al. 1999). In these early studies, serum levels of TNF-α and soluble TNF receptors (released after ligand binding) were significantly elevated in HCV infected patients compared with healthy controls.

### *IL-17*

IL-17 is a pro-inflammatory cytokine produced by a unique subset of $CD4^+$ T cells, termed Th17 cells. IL-17 plays numerous immune regulatory functions including the recruitment, activation and migration of neutrophils. Th17 cells have increased frequency in the liver of HCV infected patients and recent evidence suggests a critical role for the chemokine ligand CXCR3 in their recruitment (Oo et al. 2012; Billerbeck et al. 2010).

In my study, the increase in IL-17 production by $CD4^+$ T cells observed during therapy might be theoretically be explained by viral suppression causing a decrease in HCV non-structural protein 4 (NS4) – NS4 has been shown to induce TGF-B and IL-10 by monocytes, 2 cytokines that down-regulate production of IL-17 by $CD4^+$ T cells (Rowan et al. 2008). In support of this, although it failed to reach statistical significance the median increase in IL-17 producing $CD4^+$ T cells was 1.45% in patients who achieved an RVR compared with 0.36% in those who did not ($p = 0.32$) (Figure 4-29).

### *Interferon gamma*

Interferon gamma (IFN-γ) is the only human class II interferon. Its expression by $CD4^+$ T cells, $CD8^+$ T cells and NK cells is typically induced by exogenous stimuli like viral infection or foreign antigen. IFN-γ can directly inhibit viral replication by a number of mechanisms including: activation of intracellular RNAases; inhibition of viral protein synthesis (RNA-activated protein kinase) and blocking transport of viral ribonucleoproteins (Cheney et al.

2002; Samuel 2001). It also has immunomodulatory and anti-tumor properties. For example, it causes cells to increase MHC class I and II expression, and promotes undifferentiated T cells to become Th1 cells by upregulating the transcription factor T-bet (Mach et al. 1996; Sen 2001).

The increased capacity for IFN-γ production by T cells and NK cells in chronic HCV infection is surprising, as is the subsequent decline in its production during the first 28 days of therapy. HCV has been shown, at least in vitro, to directly inhibit IFN-γ production by hepatoma cells (Huh-T7) through degradation of STAT1 (Lin et al. 2005).

### *IL-2*

IL-2 is mainly produced by $CD4^+$ T cells following antigen-specific activation, but it is also produced to some extent by $CD8^+$ T cells and NK cells. It plays a central role in the response of the adaptive immune system to pathogens by; i) inducing proliferation of antigen specific T cells, ii) immune control through Treg production and iii) inducing the development of efficient memory T cell responses (Serti et al. 2010).

In 2005, using HCV-specific peptides, Semmo and colleagues demonstrated impaired IL-2 production by HCV specific T cells in patients with CHC compared to those who had previously resolved infection (Semmo, Day, et al. 2005b). It should be noted that the HCV specific T cell population comprises only a small proportion of circulating T cells in HCV infection and HCV specific T cell production of IL-2 production may well not reflect that of the global T cell population. More recently Petrovic and colleagues demonstrated that the HCV envelope protein 2 (HCV E2) attenuates IL-2 production by PBMCs at the level of secretion by impairing translocation of a protein kinase essential for its release (Petrovic et al. 2011). With this finding in mind, it is surprising that (as they have for TNF-α) studies

have also shown elevated plasma levels of IL-2 in patients with CHC (Bozkaya et al. 2000). It is possible that this may simply reflect a chronic inflammatory state induced by persistent infection. However, my finding of an increase in the functional reserve of PBMCs to produce this cytokine, suggests another mechanism may be at play that warrants further investigation.

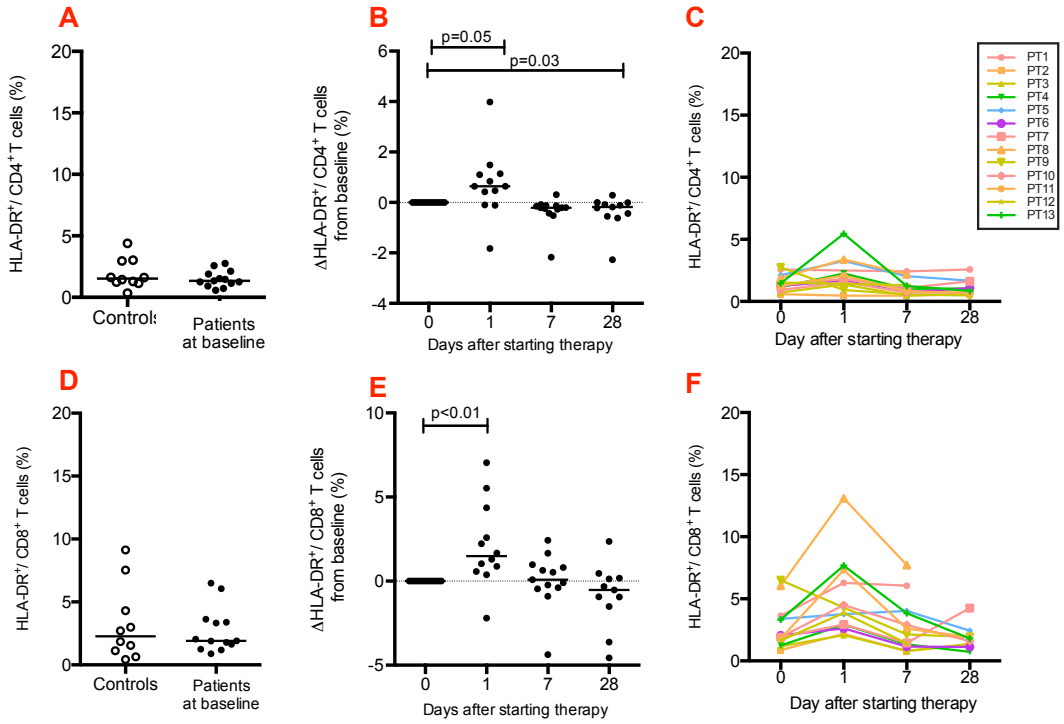
## 4.10 KEY QUESTION 5 – Does HCV infection or PEG-IFN/ribavirin therapy cause lymphocyte activation?

Surface expression of HLA-DR, CD38 and CD69 were evaluated as markers of lymphocyte activation. An example for the gating strategy used to define HLA-DR$^+$ cells is given in Appendix 13.

### 4.10.1 HLA-DR EXPRESSION

There was no difference in HLA-DR expression by lymphocytes comparing HCV infected patients and healthy controls (Figure 4-32). One day after commencing PEG-IFN/ribavirin, CD8$^+$ T cells expressing HLA-DR increased by a median of 1.5% ($p<0.01$), with a similar trend for CD4$^+$ T cells (+0.6%, $p = 0.05$). At later time-points, HLA-DR expression by all subsets gradually decreased, although this was only reached significance for CD4$^+$ T cells (-14.1%, median change, $p = 0.03$).

Figure 4-32; HLA-DR expression – baseline and longitudinal changes during therapy in HCV infected patients compared with healthy controls



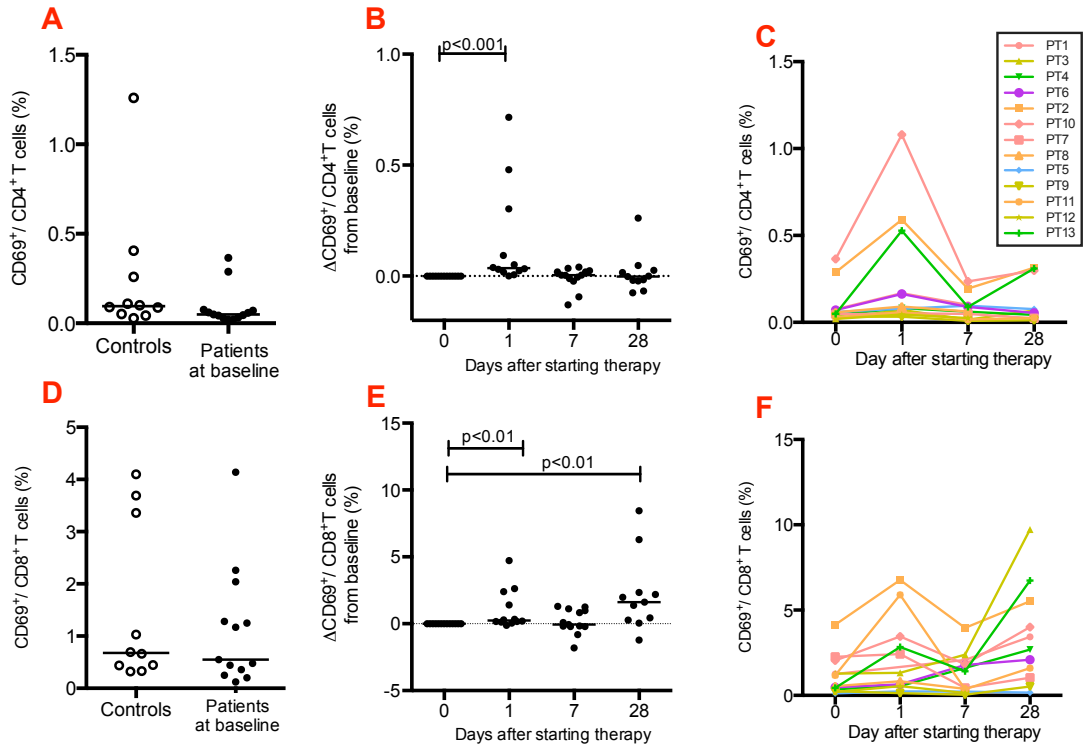

Top row: $CD4^+$ T cells. Bottom row: $CD8^+$ T cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

### 4.10.2 CD69 EXPRESSION

An example of $CD69^+$ gating is given in Appendix 14. Here, due to the absence of distinct positive and negative cell populations, gating position was originally defined using an FMO panel and then kept constant across all samples.

At baseline, no significant difference between patients and controls was observed (Figure 4-33). Between day 0 and day 1, CD69 expression by $CD4^+$ and $CD8^+$ T cells increased by medians of 0.04% ($p < 0.001$) and 0.23% ($p < 0.01$) respectively. Over the following 28 days, unlike HLA-DR, CD69 increased on $CD8^+$ T cells by a median of 1.6% ($p < 0.01$).

Figure 4-33; Longitudinal changes in CD69 expression during therapy in HCV infected patients compared with healthy controls

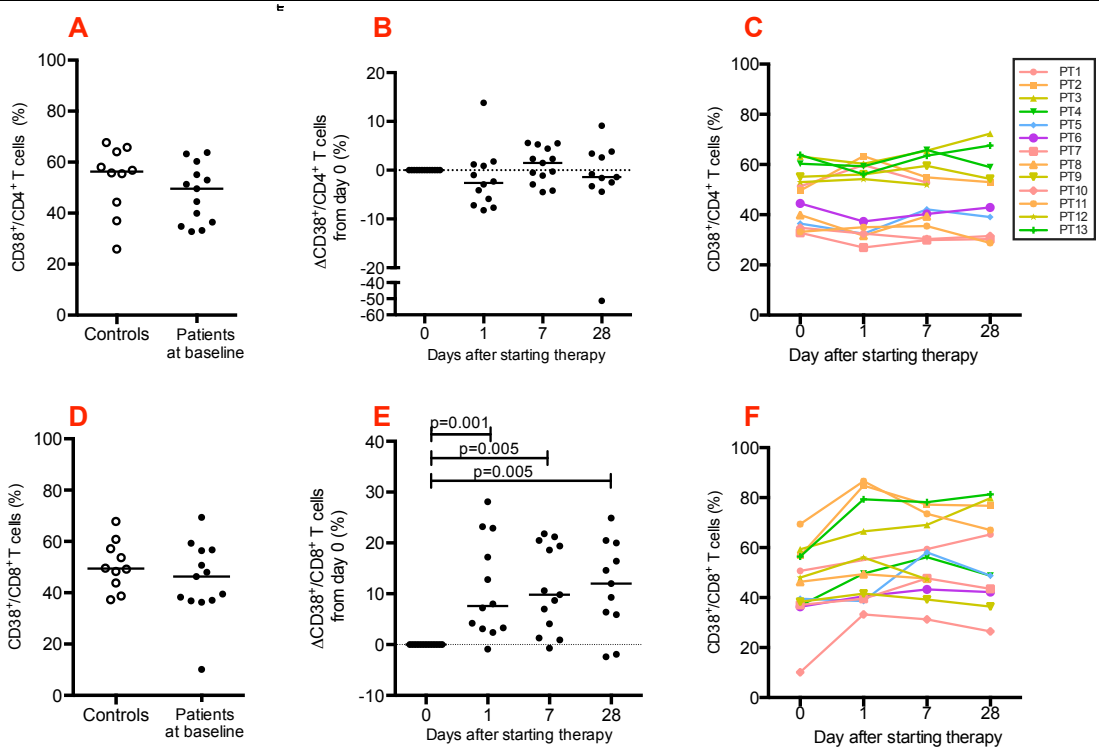



Top row: $CD4^+$ T cells. Bottom row: $CD8^+$ T cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

### 4.10.3 CD38 EXPRESSION

There was no difference in CD38 expression by $CD4^+$ or $CD8^+$ T cells comparing HCV infected patients and healthy controls. Although $CD4^+$ T cell CD38 expression remained static during therapy, it increased on $CD8^+$ T cells by a median of 12.0% by day 28 (p = 0.005).

Figure 4-34; Longitudinal changes in CD38 expression during therapy in HCV infected patients compared with healthy controls




Top row: $CD4^+$ T cells. Bottom row: $CD8^+$ T cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

## 4.10.4 DISCUSSION RELATING TO LYMPHOCYTE ACTIVATION

A summary of significant results related to this subsection is presented in Table 4-7.

Table 4-7; Significant baseline differences and longitudinal changes in circulating lymphocyte activation during HCV therapy

| | | Healthy control | | Patient at baseline | Day 1 | | Day 28 | |
|---|---|---|---|---|---|---|---|---|
| **Cell subsets** | | **% of respective subset** | | **% of respective subset** | **Δ% of respective subset from baseline** | | **Δ% of respective subset from baseline** | |
| **Activation status** | | | | | | | | |
| HLA-DR | $CD4^+$ | | | 1.3 | ↑ 0.6 | p = 0.05 (ns) | ↓ 0.18 | p = 0.03 |
| | $CD8^+$ | | | 1.9 | ↑ 1.5 | p < 0.01 | | |
| CD69 | $CD4^+$ | | | 0.05 | ↑ 0.04 | p < 0.001 | | |
| | $CD8^+$ | | | 0.56 | ↑ 0.2 | p < 0.01 | ↑ 1.6 | p < 0.01 |
| CD38 | $CD4^+$ | | | 49.6 | | | | |
| | $CD8^+$ | | | 46.3 | ↑ 7.6 | p< 0.01 | ↑ 12.0 | p = 0.005 |

In the healthy control, day 1 and day 28 columns only values significantly different from HCV infected patients at baseline (shaded blue) are listed. Markers evaluated where no difference at baseline, or change during therapy was observed, are not included. P values for day 1 and day 28 are calculated using Wilcoxon signed rank test for matched pairs, and healthy control p values calculated using the Mann-Whitney U test. Values given are absolute % values of the respective cell subset.

Taken together, the changes in HLA-DR, CD69 and CD38 expression indicate that PEG-IFN/ribavirin therapy activates circulating lymphocytes – transiently in the case of $CD4^+$ T cells, and more persistently in the case of $CD8^+$ T cells.

### *HLA-DR*

HLA-DR is a MHC-class II receptor that serves as a ligand for $CD4^+$ T cells and acts to present exogenous processed peptide antigens to these cells in its ligand groove. It is therefore most abundantly found on antigen presenting cells such as dendritic cells, B cells and macrophages. HLA-DR is nevertheless present on a small proportion of $CD4^+$ and $CD8^+$ T cells and its expression is up-regulated when these cells are activated (Salgado et al. 2002; Moriya et al. 1987).

Although my data show no difference in lymphocyte HLA-DR expression between HCV infected patients and healthy individuals, there is some evidence that HCV infection may inhibit HLA-DR expression as a means of subverting the host immune response (Saito et al.

2008). In addition, early in-vitro work by Paquette and colleagues showed up-regulation of HLA-DR on dendritic cells exposed to IFN-α suggesting therapy may rescue this impaired response (Paquette et al. 1998). In contrast, a study in 2000 found elevated serum levels of soluble serum HLA-DR in 40 patients with CHC compared with healthy controls and HLA-DR levels decreased in the first 2 weeks of PEG-IFN therapy, and remained low in those who cleared virus (Hosoi et al. 2000).

My results suggest PEG-IFN increases HLA-DR expression by $CD8^+$ and probably $CD4^+$ T cells within 24 hours of administration but this effect is transient as expression subsequently returns to baseline at day 7. It is possible that activation of T cells occurs cyclically following each PEG-IFN dose but this cannot be concluded from the available data.

### *CD69*

CD69 is a transmembrane protein detected in vivo on small subsets of circulating T and B cells. It has been well studied as a activation marker and its transcription is induced by many stimuli including IFN-α (Atzeni et al. 2002). Evidence suggests that CD69 may play a role in lymphocyte sequestration by lymphoid organs and this is supported by the observation that $CD69^+$ T cells are rare in blood compared with lymphoid tissues and inflammatory site (Testi et al. 1994; Sánchez-Mateos et al. 1989).

Work by Shiow and colleagues demonstrated surface CD69 forms complexes with, and negatively regulates sphingosine 1-phosphate receptor-1 (S1P1) a molecule essential for lymphocyte egress from lymphoid tissue (Shiow et al. 2006). Furthermore, the IFN-α/βa inducer polyinosine polycytidylic acid (poly(I:C)) was shown to up-regulate CD69

expression. When this process occurs locally it increases lymphocyte numbers in the responding lymphoid organ and when it occurs systemically it can lead to a decrease in circulating lymphocytes through sequestration.

Clearly therefore, CD69 mediated lymphocyte redistribution may contribute to lymphopenia during PEG-IFN/ribavirin therapy. In the THIRST study, the small but significant increase in CD69 expression 1 day after commencing therapy (and in the case of $CD8^+$ T cells, on day 28), at least provides in-vivo evidence that IFN-α up-regulates CD69 expression. If CD69 does regulate lymphocyte trapping, we might expect to find only minimal changes in circulating T cell expression of CD69 because those cells expressing high levels would be theoretically be sequestered.

### *CD38*

CD38 is a transmembrane glycoprotein whose expression is under complex control and has been used in the study of T and B cell activation and differentiation. Changes in CD38 expression on T cells coincides with 2 critical events during T cell ontogenesis; T cells lose their surface CD38 molecules during the acquisition of antigenic specificity and intrathymic negative selection, while mature T cells gain expression of CD38 in response to various activating stimuli (Shubinsky & Schlesinger 1997).

A 2009 study by Gonzalez and colleagues evaluated CD38 expression in 11 HCV infected patients during PEG-IFN/ribavirin therapy compared with 21 healthy controls (Gonzalez et al. 2009). The earliest time-point assessed was 4 weeks after commencing therapy. Unlike my data, CD38 expression was elevated at baseline on $CD4^+$ T cells in HCV infected patients and decreased after 4 weeks therapy. Meanwhile, CD38 expression on $CD8^+$ T

cells increased over 4 weeks therapy, before decreasing on both $CD4^+$ and $CD8^+$ T cells over the subsequent 12 weeks.

There are clear differences between my data and that of Gonzalez. Importantly, $CD38^+$ expression was significantly lower in all patients in the Gonzalez trial compared with my data – eg. ~3-5% of $CD4^+$ and $CD8^+$ T cells in healthy controls compared with 53% of $CD4^+$ and 50% of $CD8^+$ T cells in my healthy controls. Reported levels of CD38 expression on T cells vary considerably across studies with those reported by Gonzalez falling at the lower end of this spectrum (eg. Casellie; 25% of $CD8^+$ cells and Lenkei; 62% of $CD8^+$ T cells) (Caselli et al. 1999; Lenkei et al. 1998). Indeed, levels as high as 90% of $CD8^+/CD38^+$ T cells have been reported in some studies (Tiba et al. 2011). The reason for these differences are unclear but may relate to different affinities of CD38 antibody clones used to assess expression.

## 4.11 KEY QUESTION 6 – Does HCV infection or PEG-IFN/ribavirin therapy alter the differentiation state of circulating lymphocytes?

Lymphocyte differentiation was evaluated using the surface markers CCR7, CD45RA, CD57 and CD62L.

### 4.11.1 MEMORY PHENOTYPES: CCR7/45RA

The same gating strategy to define memory subsets that was used in the HCV003 trial was used to define memory subsets in the THIRST trial (see Figure 3-18, page 133). Example FACS plots demonstrating these gating strategies are presented in Figure 4-35.

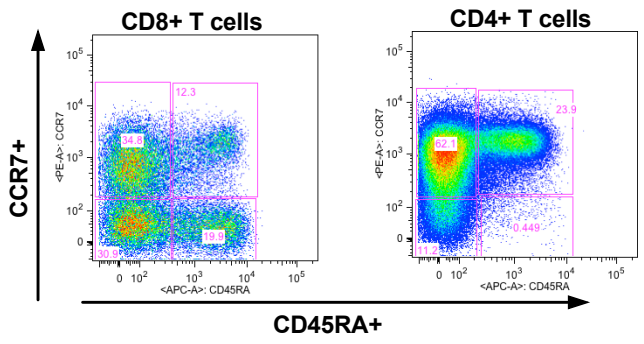
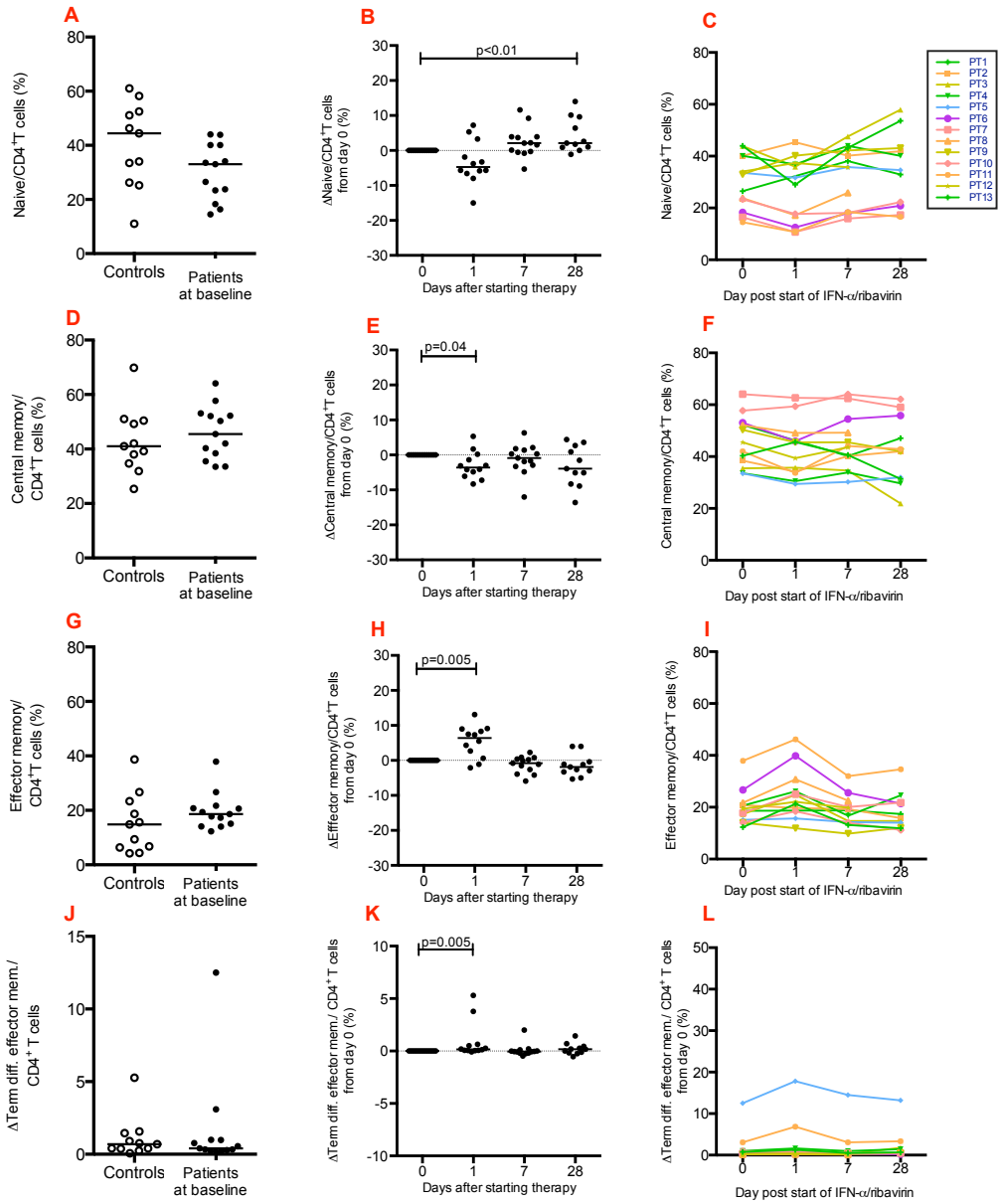



Figure 4-35; Gating strategy for defining cell differentiation status based on CCR7 & CD45RA expression combined

The plots have first been gated on $CD3^+$ lymphocytes. See Figure 3-18 for memory gating subset definitions, page 133.

At baseline, there was no difference in the differentiation status of $CD4^+$ (Figure 4-36) or $CD8^+$ (Figure 4-37) T cell subsets comparing HCV infected patients to healthy controls (based on CCR7/CD45RA co-expression). A trend towards smaller proportions of circulating naïve $CD4^+$ T cells in patients compared with controls was not significant (33.0% vs. 44.4%, $p = 0.07$).

Figure 4-36; Baseline differences and longitudinal changes in CD4+ memory subsets during therapy in HCV infected patients



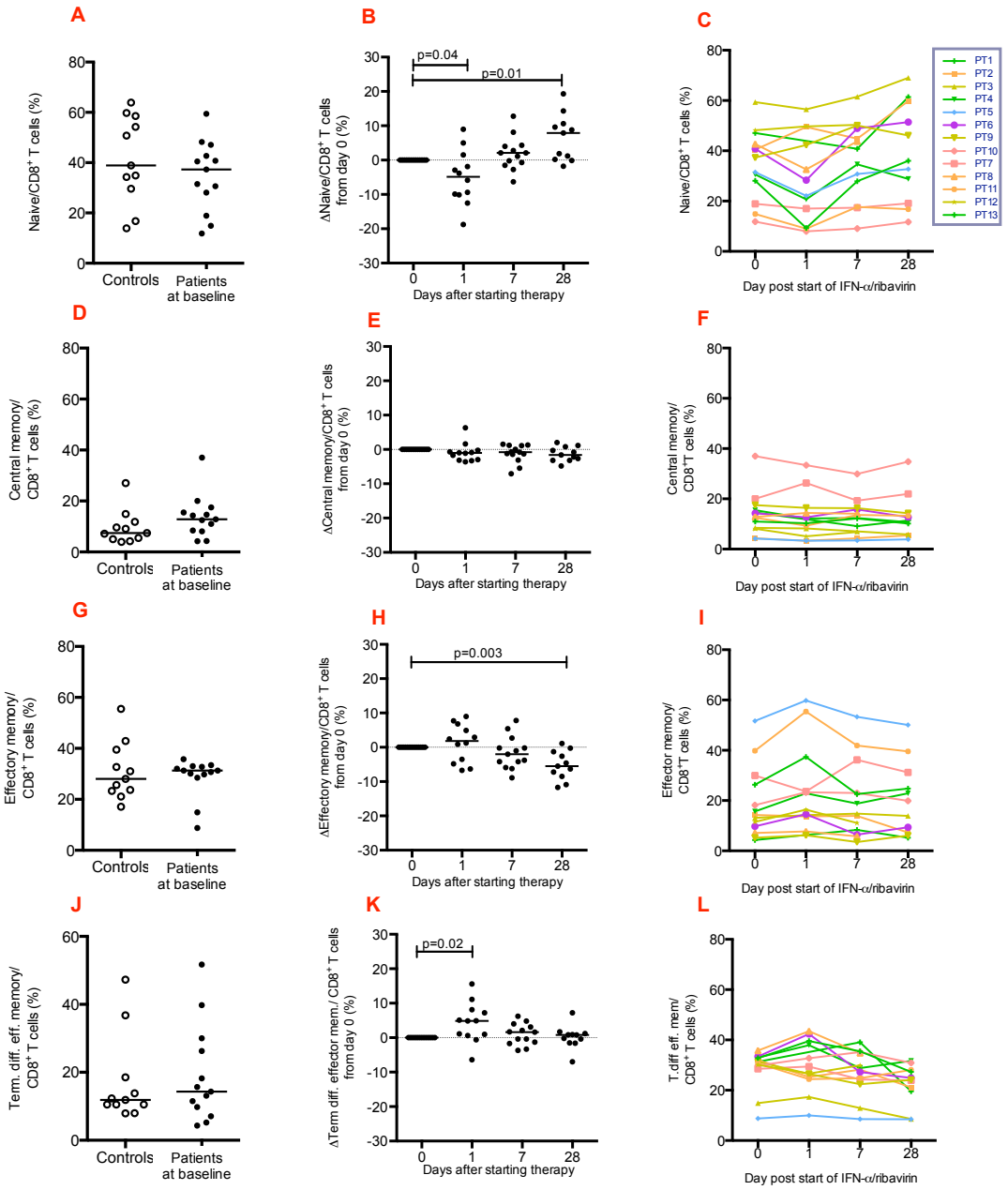

Each row represents a different CD4+ T cell memory subset: 1st row; naïve, 2nd row; central memory, 3rd row; effector memory, bottom row; terminally differentiated effector memory cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

One day after commencing therapy, the proportion of circulating terminally differentiated effector memory T cells increased significantly for both CD4+ and CD8+ T cell populations

(CD4; +5.35%, p = 0.005 and CD8; +1.8%, p = 0.02, median changes). Meanwhile, the number of circulating naïve T cells decreased in both groups, although this was only significant for $CD8^+$ T cells (median decrease 4.9%, p = 0.04). In addition, amongst the $CD4^+$ T cell population only, central memory T cells decreased by a median of 3.6% (p = 0.04), while effector memory T cells increased by a median of 6.4% (p < 0.01). These changes were all transient and memory populations had returned to baseline at day 7.

By day 28 of therapy, an increase in the proportion of circulating naïve T cells was observed in both $CD4^+$ and $CD8^+$ T cell populations (median increases 2.1% (p < 0.01) and 10.6% (p = 0.01) respectively). A significant decrease in effector memory T cells was isolated to the $CD8^+$ T cell population (median decrease 5.5%, p < 0.01).

Figure 4-37; Baseline differences and longitudinal changes in CD8+ memory subsets during therapy in HCV infected patients

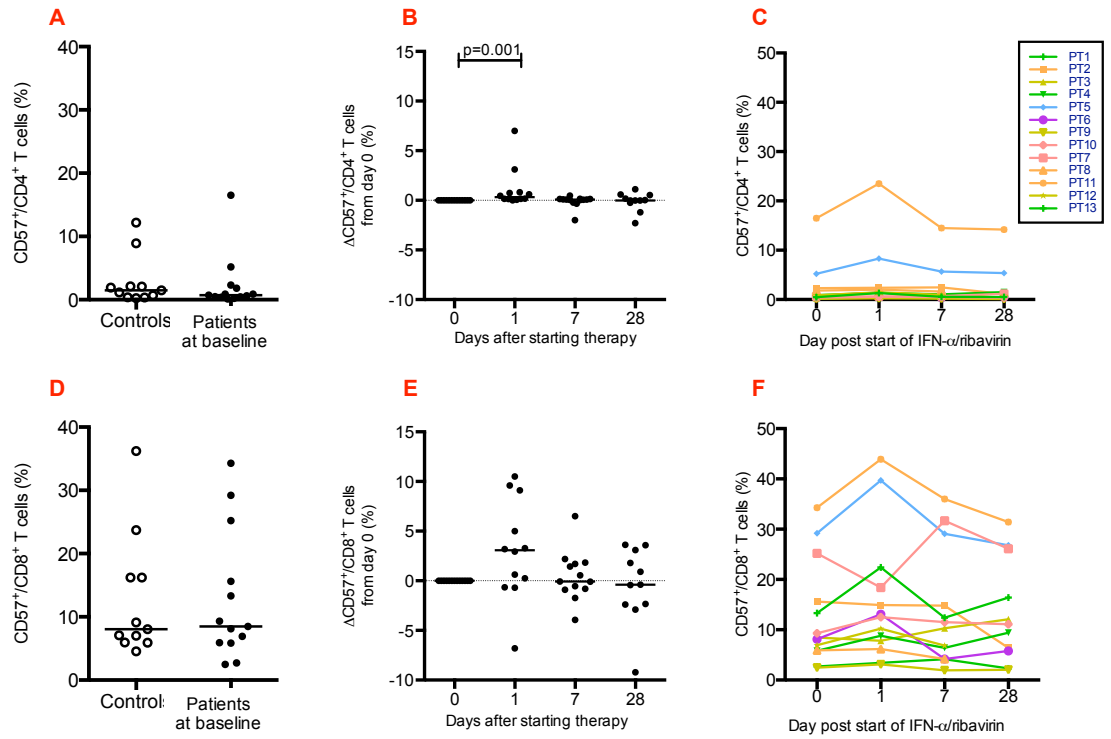



Each row represents a different CD8+ T cell memory subset: 1st row; naïve, 2nd row; central memory, 3rd row; effector memory, bottom row; terminally differentiated effector memory cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

## 4.11.2 CD57 EXPRESSION

There was no difference in CD57 expression on $CD4^+$ or $CD8^+$ T cells between HCV infected patients and healthy controls. Between day 0 and day 1 of treatment, the proportion of $CD4^+$ T cells expressing CD57 increased by a median of 0.32% (p = 0.001) and a similar trend was observed for $CD8^+$ T cells (+3.10%, p = 0.08). CD57 expression thereafter returned to baseline and remained static out to day 28 (Figure 4-38).

Figure 4-38; Longitudinal changes in CD57 expression during therapy in HCV infected patients compared with healthy controls




Top row: $CD4^+$ T cells. Bottom row: $CD8^+$ T cells. Left column: patients vs. healthy controls. Middle column: values are normalised against day 0 for each individual patient to demonstrate longitudinal change from baseline. Right column: coloured lines connect individual patient samples across time. Horizontal lines depict median values at each time-point.

It is possible to evaluate CD57 expression on T cells in more detail according to memory phenotype (CCR7/CD45RA) (Appendix 15). No difference was observed between patients with CHC and controls in CD57 expression by any T cell population subset.

### 4.11.3 CD62L EXPRESSION

No difference in CD62L expression by $CD4^+$ or $CD8^+$ T cells was observed between controls and patients with CHC at baseline, or during the course of PEG-IFN/ribavirin therapy (Appendix 16).

## 4.11.4 DISCUSSION RELATING TO LYMPHOCYTE DIFFERENTIATION DURING HCV THERAPY

A summary of significant results related to this subsection is presented in Table 4-8.

Table 4-8; Significant baseline differences and longitudinal changes in the differentiation status of circulating lymphocytes during HCV therapy

| | | Healthy control | | Patient at baseline | Day 1 | | Day 28 | |
|---|---|---|---|---|---|---|---|---|
| **Cell subsets** | | **% of respective subset** | | **% of respective subset** | **Δ% of respective subset from baseline** | | **Δ% of respective subset from baseline** | |
| **Differentiation status** | | | | | | | | |
| T. diff. eff. mem | $CD4^+$ | | | 0.4 | ↑ 0.2 | p = 0.005 | | |
| | $CD8^+$ | | | 14.3 | ↑ 4.9 | p = 0.02 | | |
| Effector memory | $CD4^+$ | | | 18.6 | ↑ 6.4 | p < 0.01 | | |
| | $CD8^+$ | | | 31.3 | | | | |
| Naïve | $CD4^+$ | | | 33.0 | | | ↑ 2.1 | p < 0.01 |
| | $CD8^+$ | | | 37.3 | ↓ 4.9 | p = 0.04 | ↑ 10.6 | p < 0.01 |
| Central memory | $CD4^+$ | | | 45.5 | ↓3.6 | p = 0.04 | | |
| | $CD8^+$ | | | 12.8 | | | | |
| $CD57^+$ | $CD4^+$ | | | 0.7 | ↑0.32 | p < 0.01 | | |
| | $CD8^+$ | | | 8.5 | | | | |

In the healthy control, day 1 and day 28 columns only values significantly different from HCV infected patients at baseline (shaded blue) are listed. Markers evaluated where no difference at baseline, or change during therapy was observed, are not included. P values for day 1 and day 28 are calculated using Wilcoxon signed rank test for matched pairs, and healthy control p values calculated using the Mann-Whitney U test. Values given are absolute % values of the respective cell subset. T. diff. eff. mem = terminally differentiated effector memory cells.

The combined expression pattern of the surface markers CCR7 and CD45RA, has been widely used to define the memory phenotype of T cells as shown in Figure 3-18. C-C Chemokine Receptor 7 (CCR7) modulates homing of T cells and dendritic cells to lymph nodes where it drives antigen specific activation of T cells by promoting co-location with APCs. After priming, T cells modulate CCR7 expression - for example, down-regulation of expression permits egress of effector memory T cells from the lymph node (Förster et al. 2008). CD45RA on the other hand, is 1 isoform of a family of CD45 molecules. The CD45 protein is a transmembrane protein tyrosine phosphatase (PTP) involved in regulation of T- and B-cell receptor signaling. Naïve T lymphocytes are usually positive for CD45RA in comparison to activated and memory T cells which express the shortest CD45 isoform CD45RO.

I found no significant difference in either $CD4^+$ or $CD8^+$ T cell differentiation amongst patients with HCV infection compared to healthy controls. During PEG-IFN/ribavirin therapy an early rise in terminally differentiated T cells is followed later by an increase in circulating naïve $CD4^+$ and $CD8^+$ T cells after 4 weeks.

The increase in CD57 expression on $CD4^+$ T cells after 1 day of PEG-IFN/ribavirin therapy is in keeping with the increase in terminally differentiated effector memory cells ($CCR7^-$/$CD45RA^+$) observed because these cells express high levels of CD57. CD57 was originally discovered on human NK cells but has more recently been shown to be expressed on T lymphocytes where it is considered to be a marker of senescence ('clonal exhaustion') (Brenchley et al. 2003).

A 2010 study by Lee and colleagues used CD45RA and CD57 expression to define memory subpopulations in 34 patients with CHC (S. Lee et al. 2010) because no CCR7 antibody was commercially available at the time. They too, found no difference between healthy controls and HCV infected patients. PBMCs were collected from 19 patients once during therapy in this trial (at no set time-point, median 5 months therapy). Similar to my data, proportions of naive $CD8^+$ T cells increased during therapy. However, naïve $CD4^+$ T cell proportions remained static.

In 2007, Golden-Mason and colleagues explored CD57 expression on HCV specific T cells and found increased expression in patients with CHC infection compared with those who had previously resolved infection (Golden-Mason et al. 2007). No data evaluating CD57 expression on bulk T cell populations is given in this study. However, another senescence

marker PD-1 expression was elevated on bulk T cell populations suggesting a global effect of HCV on T cell exhaustion.

CD62L is another marker used to define memory cell populations. It acts as a homing receptor for lymphocytes to enter secondary lymphoid organs via high endothelial venules. Naïve T cells and central memory cells should theoretically express CD62L while effector memory cells do not. The absence of a significant change in the expression of this marker during HCV therapy contrasts with the observed changes in CCR7/CD45RA expression.

## 4.12 SUMMARY DISCUSSION OF THIRST STUDY

This detailed examination of lymphocyte subpopulations in the setting of chronic HCV infection, and longitudinally during PEG-IFN/ribavirin therapy, supports previously published work and also reveals several new findings relevant to the application of a therapeutic vaccine during therapy. These findings are summarised in Table 4-9.

Key differences identified in patients with chronic HCV infection compared with healthy individuals are:

i) An increase in the proportion of circulating regulatory T cells

ii) A relative increase in circulating $CD56^{bright}$ NK cells (amongst the total NK cell population)

iii) Global alterations in lymphocyte cytokine profiles with increased capacity for IFN-γ, IL-2 and TNF-α production upon PMA/ionomycin stimulation

It is interesting to note that comparing patients with healthy controls, the increased functional reserve for cytokine production was not isolated to single lymphocyte subpopulations (eg. both $CD4^+$ T cells and NK cells produced more IFN-γ, with a similar trend observed in $CD8^+$ T cells). Why PBMCs from patients with CHC should have a greater reserve for the production of certain pro-inflammatory cytokines is unclear. Potentially alterations in the differentiation status of circulating cells, might contribute to this. Although the design of my panels did not permit subtyping of cytokine-producing cells based on their cellular differentiation, the absence of any major differences in basic memory phenotypes of circulating T cells does not support this. What other mechanisms could be at play? Had circulating Treg numbers been reduced in patients, this might have

provided some explanation, but findings were to the contrary – in keeping with other published data, there was a significant increase in circulating Tregs (Cabrera et al. 2004).

The relative increases in circulating regulatory T cells and $CD56^{bright}$ cells in HCV infected patients are not novel findings. Nevertheless, they provide evidence that both adaptive and innate immunity is altered by chronic HCV infection, and these changes might well contribute to the reduced immunogenicity of ChAd3-NSmut/MVA-NSmut vaccination observed in the HCV003 trial.

It is possible, that experiment procedural differences relating to functional stimulation might have contributed to the observed differences in cytokine production between healthy controls and HCV infected individuals. In particular, it should be noted that healthy volunteer samples were PMA/ionomycin stimulated and analysed in experiments separate to those performed on patient samples. For this reason, the observed differences between healthy controls and HCV infected patients in relation to cytokine production will be validated in a larger cohort.

Longitudinal analysis of PBMCs during the first 28 days of PEG-IFN/ribavirin therapy indicated several key treatment-induced changes:

i) Although therapy causes lymphopenia in most patients, only minor changes in the relative subpopulations of lymphocytes and their differentiation status occurs.

ii) Circulating lymphocytes that express the liver homing chemokine receptors (CXCR3 and CCR6) decrease transiently.

iii) Over 28 days, the capacity of PMA/ionomycin stimulated lymphocytes to produce IFN-γ decreases, and this is complemented by increases in TNF-α and IL-17 production.

iv) T cells are activated (particularly $CD8^+$ T cells), as judged by HLA-DR, CD38 and CD69 expression.

Taken in the context of the substantial decrease (39%) in the total number of circulating lymphocytes between day 0 and day 28, the relatively minor proportional changes in subpopulations suggests that the mechanism of treatment-induced lymphopenia affects these subsets equally. Although bone marrow suppression might provide some explanation for this global change (Schmid 2005), relative changes in specific subpopulations, such as an increase in $CD56^{bright}$ NK cells are less likely to be explained by this broad sweeping mechanism.

One alternate explanation for the changes observed in circulating lymphocyte subset proportions is that of therapy induced, chemokine-mediated lymphocyte sequestration. If PEG-IFN therapy causes release of CXCR3- or CCR6-binding chemokines, this might explain lymphopenia to some extent. The early preferential decrease in lymphocytes expressing these chemokine receptors observed in my study provides indirect evidence to support this theory. Importantly, analysis of chemokine receptor co-expression indicates that day 1 changes are likely to be driven by CXCR3-binding chemokines because only circulating $CCR6^+$ T cells that co-express CXCR3 decrease.

Recent work has demonstrated a critical role for CXCR3-binding chemokines in hepatic recruitment of regulatory T cells and Th17 cells (Oo et al. 2012; Oo et al. 2010). If CXCR3-

binding chemokines are playing an important role in early lymphopenia during PEG-IFN/ribavirin therapy, why then did I find no decrease in circulating Tregs, and an increase (rather than a decrease) in IL-17 producing $CD4^+$ T cells? Unfortunately, the relative expression of CXCR3 by Tregs and Th17 cells could not be compared with other lymphocyte subsets in the THIRST study, due to the design of flow cytometry panels. It is however, clear CXCR3 mediates recruitment of other T cell subsets such as effector T cells (Curbishley et al. 2005) and therefore, proportional changes in Tregs and Th17 cells relative to other lymphocyte subsets might not be observed. Furthermore, changes in IL-17 production might relate to viral suppression mediated changes in other immunomodulatory cytokines, rather than lymphocyte redistribution (see page 197).

## 4.13 CONCLUSION

Early during therapy, temporal changes in circulating lymphocyte subpopulations, function, activation and differentiation status are observed that may impact on the successful application of a therapeutic vaccine for HCV infection. These findings highlight the importance of evaluating vaccine immunogenicity in patients not receiving concurrent PEG-IFN/ribavirin therapy, and also considering the impact of vaccine timing in those who are. Furthermore, this work raises several new questions relating to the baseline functional reserve of lymphocytes in HCV infected patients, and perhaps most intriguingly the possibility that therapy induces chemokine-mediated lymphocyte sequestration. In the next chapter, this novel finding will be carried forward with the aim of determining whether CXCR3 mediated sequestration is a key mechanism in HCV therapy induced lymphopenia.

Table 4-9; Significant baseline differences and longitudinal changes in circulating lymphocytes during HCV therapy

| | | Healthy control | | Patient at baseline | Day 1 | | Day 28 | |
|---|---|---|---|---|---|---|---|---|
| **Cell subsets** | | **% of respective subset** | | **% of respective subset** | **Δ% of respective subset from baseline** | | **Δ% of respective subset from baseline** | |
| $CD3^+$ lymphocytes/PBMCs | | | | 67.1 | | | ↑ 6.0 | p < 0.01 |
| | $CD4^+$ | | | 68.4 | | | ↑ 2.1 | p = 0.04 |
| | $CD8^+$ | | | 24.8 | ↓ 1.0 | p = 0.02 | | |
| | $CD161^{bright}$ | 13.1 | p < 0.01 | 4.0 | ↓ 0.5 | p = 0.04 | | |
| | Tregs | 2.5 | p = 0.02 | 4.2 | | | | |
| B cells/PBMCS | | | | 9.2 | | | ↓ 3.3 | p = 0.02 |
| $NK^{bright}/NK^{dim}$ | | 5.3 | p = 0.03 | 11.4 | | | ↑ 3.6 | p < 0.01 |
| **Activation status** | | | | | | | | |
| HLA-DR | $CD4^+$ | | | 1.3 | | | ↓ 0.18 | p = 0.03 |
| | $CD8^+$ | | | 1.9 | ↑ 1.5 | p < 0.01 | | |
| CD69 | $CD4^+$ | | | 0.05 | ↑ 0.04 | p < 0.001 | | |
| | $CD8^+$ | | | 0.56 | ↑ 0.2 | p < 0.01 | ↑ 1.6 | p < 0.01 |
| CD38 | $CD4^+$ | | | 49.6 | | | | |
| | $CD8^+$ | | | 46.3 | ↑ 7.6 | p < 0.01 | ↑ 12.0 | p < 0.01 |
| **Differentiation status** | | | | | | | | |
| T diff. eff. mem | $CD4^+$ | | | 0.4 | ↑ 0.2 | p < 0.01 | | |
| | $CD8^+$ | | | 14.3 | ↑ 4.9 | p = 0.02 | | |
| Effector memory | $CD4^+$ | | | 18.6 | ↑ 6.4 | p < 0.01 | | |
| | $CD8^+$ | | | 31.3 | | | | |
| Naïve | $CD4^+$ | | | 33.0 | | | ↑ 2.1 | p < 0.01 |
| | $CD8^+$ | | | 37.3 | ↓ 4.9 | p = 0.04 | ↑ 10.6 | p < 0.01 |
| Central memory | $CD4^+$ | | | 45.5 | ↓3.6 | p = 0.04 | | |
| | $CD8^+$ | | | 12.8 | | | | |
| $CD57^+$ | $CD4^+$ | | | 0.7 | ↑0.32 | p < 0.01 | | |
| | $CD8^+$ | | | 8.5 | | | | |
| **Intracellular cytotoxicity stains** | | | | | | | | |
| Granzyme $B^+$ $CD8^+$ T cells | | | | 14.7 | ↑ 4.7 | p = 0.03 | | |
| **Homing marker** | | | | | | | | |
| CXCR3 | $CD4^+$ | 7.4 | p < 0.01 | 16.3 | ↓ 3.6 | p < 0.01 | | |
| | $CD8^+$ | | | 37.5 | ↓ 11.5 | p = 0.03 | | |
| | B cells | 30.6 | p < 0.01 | 53.2 | ↓ 20.1 | p < 0.01 | ↓12.3 | p = 0.02 |
| | NK cells | | | 11.2 | ↓ 2.3 (day 7) | p < 0.01 | | |
| CCR6 | $CD4^+$ | | | 7.1 | ↓ 1.7 | p = 0.04 | ↓ 3.6 | p = 0.03 |
| | $CD8^+$ | 40.2 | p < 0.0001 | 14.3 | ↓ 3.6 | p = 0.01 | | |
| | NK cells | 36.5 | p < 0.01 | 26.7 | ↓ 7.8 | p = 0.001 | ↓ 5.7 | p < 0.001 |
| **Functional changes** | | | | | | | | |
| IFN-γ | $CD4^+$ | 14.2 | p = 0.02 | 23.4 | | | ↓ 2.1 | p = 0.01 |
| | $CD8^+$ | | | 46.6 | | | ↓ 15.2 | p = 0.001 |
| | NK cells | 20.8 | p = 0.04 | 35.8 | | | ↓ 11.6 | p = 0.002 |
| IL-2 | $CD4^+$ | 11.0 | p < 0.01 | 27.0 | | | | |
| | $CD8^+$ | 7.1 | p < 0.01 | 17.0 | | | | |
| IL-17 | $CD4^+$ | | | 0.50 | ↑ 1.0 | p = 0.02 | ↑ 1.0 | p < 0.01 |
| TNF-α | $CD4^+$ | | | 25.6 | ↑ 6.9 | p < 0.01 | | |
| | $CD8^+$ | 27.0 | p = 0.03 | 36.5 | ↑ 4.0 | p < 0.01 | | |

In the healthy control, day 1 and day 28 columns only values significantly different from HCV infected patients at baseline (shaded blue) are listed. Markers evaluated where no difference at baseline, or change during therapy was observed, are not included. P values for day 1 and day 28 are calculated using Wilcoxon signed rank test for matched pairs, and healthy control p values calculated using the Mann-Whitney U test. Values given are absolute % values of the respective cell subset. T.diff. eff. Mem = terminally differentiated effector memory cells.

# Chapter 5 The role of CXCR3 in lymphocyte recruitment during HCV therapy

**Abstract accepted for poster presentation relating to this chapter and awarded travel bursary:** J.S. Halliday, N. Ramamurthy, C. Willberg, J. Ussher, J. Phillips, L. Swadling, C. Cosgrove, P. Klenerman, E. Barnes *'IP-10 release driven by interferon-α suggests a role for lymphocyte recruitment in HCV clearance',* United European Gastroenterology Week, 2012.

## 5.1 INTRODUCTION

One of the most striking changes observed in the THIRST trial (Chapter 4) was a decrease in circulating $CXCR3^+$ expressing lymphocytes 1 day after commencing PEG-IFN/ribavirin therapy. This finding suggests the possibility of chemokine-mediated lymphocyte sequestration during HCV therapy.

CXCR3 is a G-protein coupled membrane bound receptor that binds the chemokines, CXCL9 (MIG-I; monokine induced by gamma interferon), CXCL10 (or IP-10; IFN-ϒ inducible protein-10) and CXCL11 (ITAC; interferon inducible T cell alpha chemoattractant). It is widely expressed on immune cells including neutrophils and lymphocytes and is thought to be partly responsible for hepatic homing of these cells during inflammation (Moser & Loetscher 2001).

Historically, the CXCR3-binding chemokines have been thought to play no direct role in IFN-α mediated HCV clearance because only gradual declines in CXCL10 or non-specific changes in CXCL11 have been observed during HCV therapy (Butera et al. 2005). Considering the theoretically beneficial role of lymphocyte recruitment to sites of infection, one might expect increased plasma CXCL10 in patients who respond to therapy. Paradoxically however, high pre-treatment plasma levels of CXCL10 (but not CXCL11)

have actually been shown to be independently predictive of a poor response to standard therapy for CHC (Narumi et al. 1997; Apolinario et al. 2004; Butera et al. 2005). One possible explanation for this comes from recent work by Albert and colleagues demonstrating that an antagonist form of CXCL10 exists in patients with CHC infection that is not distinguished by traditional CXCL10 ELISA assays (Casrouge et al. 2011). Alternatively, high CXCL10 levels may reflect maximal pre-existing stimulation of the inflammatory cascade thereby limiting further amplification with PEG-IFN and abrogating its beneficial effect.

Rather than directing $CXCR3^+$ lymphocyte sequestration, an alternative explanation for the decrease in $CXCR3^+$ T cells during IFN-α therapy is that IFN-α may down-regulate surface expression of CXCR3 – a process which has previously been demonstrated to be rapidly induced by ligand binding (Meiser et al. 2008; Sauty et al. 2001). With this in mind, an ideal method to evaluate the possibility of CXCR3 dependent hepatic homing of lymphocytes driven by PEG-IFN/ribavirin would be to assess longitudinal liver biopsy specimens from HCV infected patients during therapy. Unfortunately, due to risk and morbidity associated with liver biopsies, this is not possible. Instead, this chapter aims to evaluate these hypotheses as outlined below:

## 5.2 PRIMARY HYPOTHESIS

Exogenously administered IFN-α drives hepatic recruitment of circulating CXCR3+ lymphocytes by induction of CXCR3-binding chemokines.

**SUMMARY OF PREVIOUS RESULTS FROM WHICH THIS HYPOTHESIS IS GENERATED:**

i) Significant decreases in circulating CXCR3+ lymphocytes are observed 1 day after commencing IFN-α therapy (Chapter 4).

ii) IFN-α therapy for HCV causes lymphopenia in a significant proportion of patients (Humphreys et al. 2012).

## 5.3 PRIMARY OBJECTIVE

To demonstrate in-vivo, that IFN-α therapy induces CXCR3-binding chemokines and this causes hepatic sequestration of lymphocytes.

## 5.4 KEY QUESTIONS

**1.** Does IFN-α induce CXCR3-binding chemokines in vivo and if so, are these linked to changes in CXCR3 expression?

**2.** If (1) is true, is the liver a source of CXCR3-binding chemokines?

**3.** Alternatively, does IFN-α cause down-regulation of CXCR3 on T lymphocytes, or induce death of this cell population?

**4.** If findings from (i-iii) support the primary hypothesis, can in-vivo enrichment of hepatic CXCR3+ lymphocytes be demonstrated in a mouse model by administration of IFN-α?

## 5.5 KEY QUESTION 1 - Does IFN-α induce CXCR3-binding chemokines in vivo, and are these linked to changes in CXCR3 expression in circulatoin?

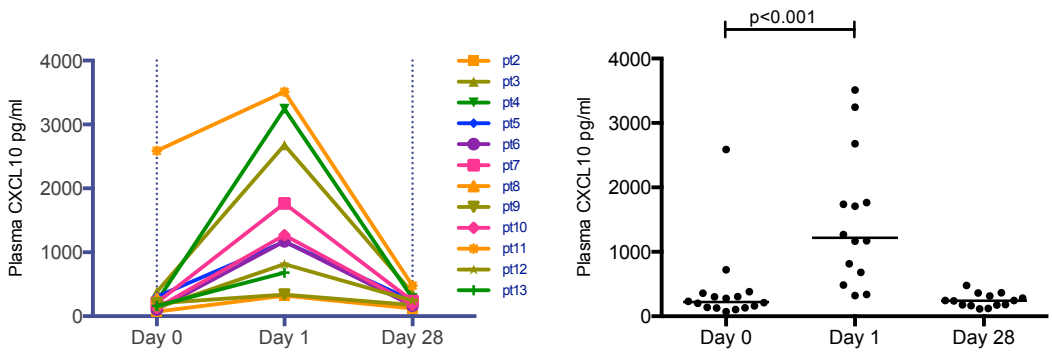
Day 0, 1 and 28 plasma samples were analysed for 11 of the patients originally enrolled in the THIRST trial. An additional 3 patients were recruited (designated patients 20, 21 and 22) for detailed analysis of longitudinal changes during PEG-IFN/ribavirin therapy. For these 3 patients, plasma samples were also collected on, and 1 day after their 2nd, 3rd and 4th PEG-IFN doses.

### 5.5.1 EVALUATION OF CXCL10 DURING PEG-IFN/RIBAVIRIN THERAPY

#### *Longitudinal changes in plasma concentration of CXCL10*

Measured by ELISA, plasma CXCL10 concentration increased significantly from a median of 221.7 to 1220.0 pg/ml between day 0 and day 1 of therapy. This increase was however, transient and levels had returned to baseline by day 28 ($p < 0.001$) (Figure 5-1).

Figure 5-1; Longitudinal plasma CXCL10 concentrations during PEG-IFN/ribavirin therapy




Right graph: dotted vertical lines represent times of PEG-IFN dosing (samples at these time points were taken prior to PEG-IFN dosing). Left graph: coloured lines join individual patient samples at each time-point. Horizontal lines represent median values.

The concentration of CXCL10 on day 0 was not predictive of the subsequent change induced by the first dose of PEG-IFN (Figure 5-2).

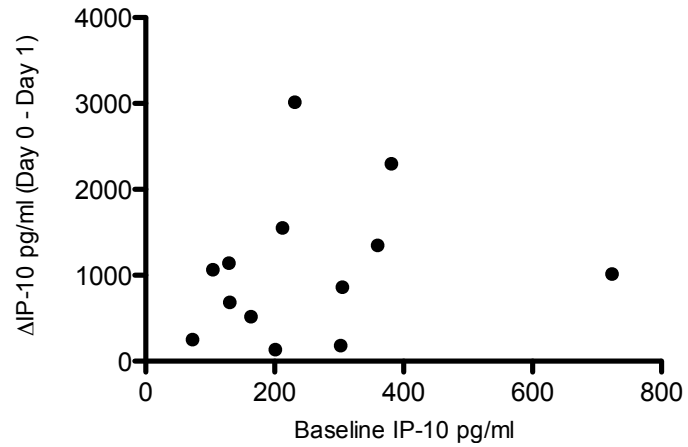
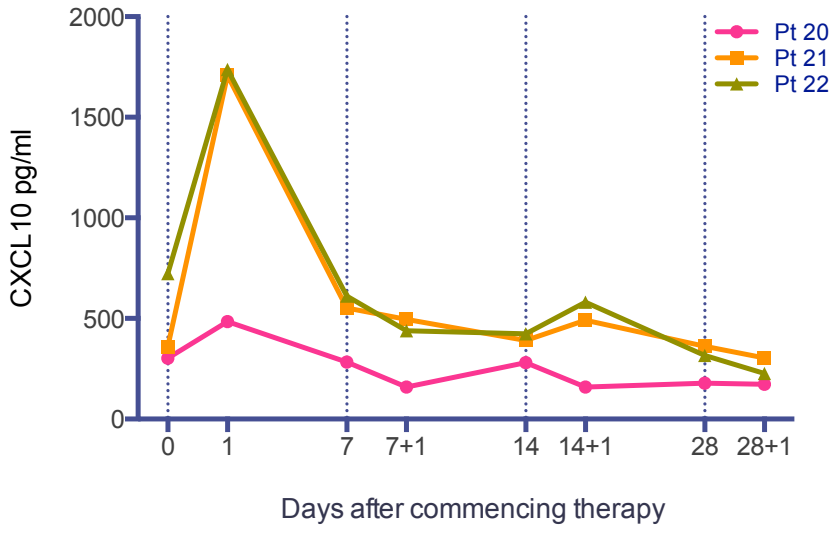



Figure 5-2; Baseline plasma CXCL10 relative to the change in CXCL10 between day 0 and day 1 of PEG-IFN/ribavirin therapy

Each point represents an individual patient value. ΔCXCL10= day 1 – day 0 concentration

Results from the 3 patients who had plasma CXCL10 determined 24 hours after the 2nd, 3rd and 4th doses of PEG-IFN are presented in Figure 5-3. Surprisingly, the increase in plasma CXCL10 observed 1 day after the 1st dose of PEG-IFN, did not recur following subsequent doses.

Figure 5-3; Longitudinal plasma CXCL10 concentrations during PEG-IFN/ribavirin therapy




Coloured lines connect individual patient samples across time. Dotted vertical lines represent times of PEG-IFN dosing (samples at these time-points were taken prior to PEG-IFN administration).

### *CXCL10 and circulating lymphocyte number*

At baseline, there was no correlation between plasma CXCL10 and total circulating lymphocyte number. However, between day 0 and day 1 of therapy, there was a suggestion that changes in circulating lymphocyte number and CXCL10 inversely correlated (Figure 5-4). It is likely this trend did not reach statistical significance because matched CXCL10 levels and lymphocyte counts on day 1 were available for analysis in only 5 patients (r = -0.26, p = 0.12).

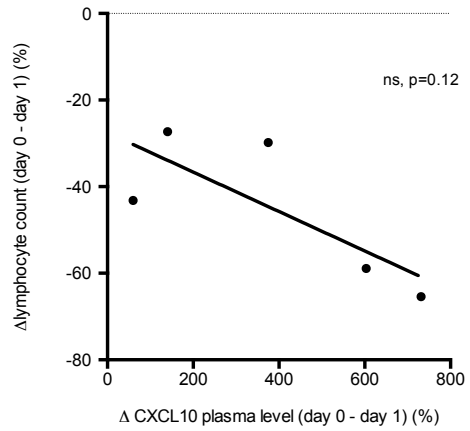



Figure 5-4; ΔCXCL10 between day 0 and day 1 of therapy compared with the percentage change in circulating lymphocyte number

X-axis: ΔCXCL10, as a proportion of the baseline concentration.
Y-axis: Δlymphocyte count, as a proportion of the baseline lymphocyte count.
Spearman correlation for non-parametric data applied for this analysis.

Further evidence to support a relationship between CXCL10 and circulating lymphocyte number comes from the longitudinal analysis of patients 20, 21 and 22. Here, just as it was for CXCL10, the relative change in lymphocyte count was greatest after the first dose of PEG-IFN, while for subsequent doses this change was relatively minor (Figure 5-5).

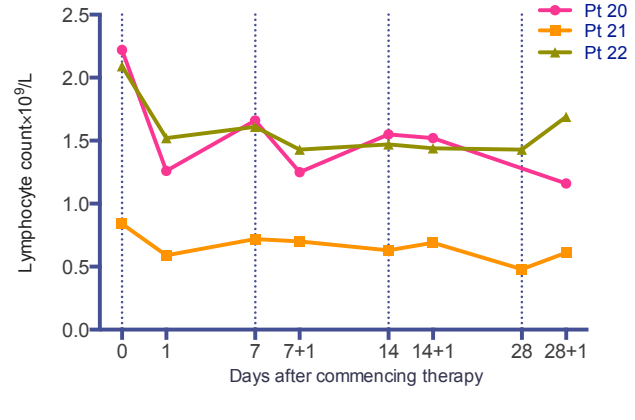


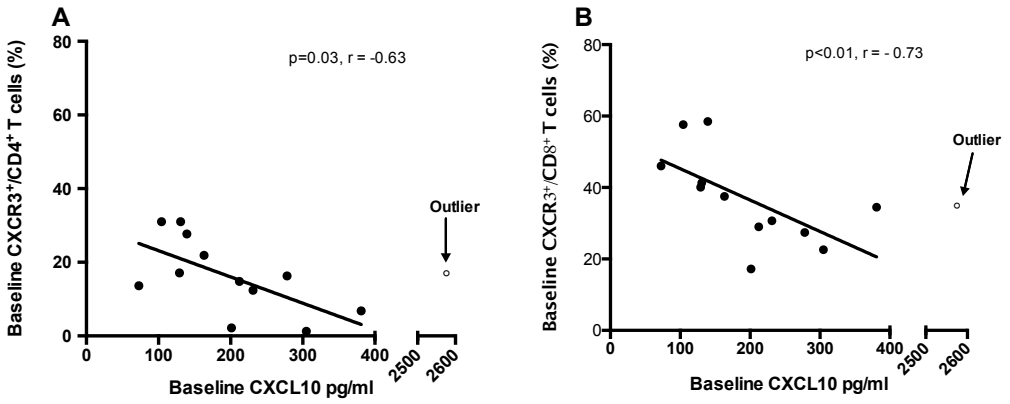

Figure 5-5; Longitudinal total circulating lymphocyte counts during PEG-IFN/ribavirin therapy

Coloured lines connect individual patient samples across time. Dotted vertical lines represent times of PEG-IFN dosing. Samples at these time-points were taken prior to PEG-IFN dosing

### *CXCL10 and CXCR3 expression*

At baseline, there was a significant correlation between plasma CXCL10 and CXCR3 expression by both $CD4^+$ and $CD8^+$ T cells (patient 11 was excluded from this analysis as an outlier: baseline CXCL10 = 2588 pg/ml compared with group mean ± 2SD = 395 ± 1210). The same trend was apparent for B cells although this failed to reach significance (p = 0.09).

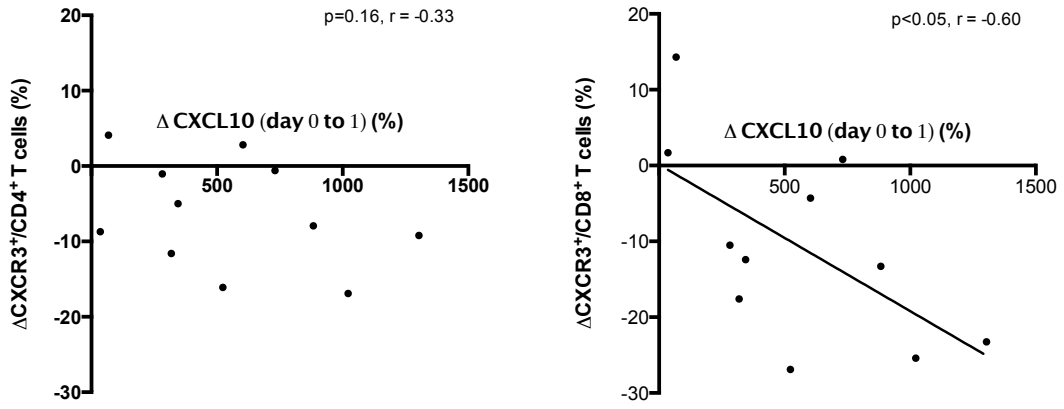
Figure 5-6; Correlation between plasma CXCL10 and CXCR3 expression by T cells at baseline




Graph A: $CD4^+$ T cells. Graph B: $CD8^+$ T cells. r calculated using Spearman coefficient for non-parametric data

Between day 0 and day 1 of therapy, there was an inverse correlation between the relative change in plasma CXCL10 and CXCR3 expression by $CD8^+$ T cells ($p = 0.03$, one-tailed, $r = -0.59$), but not for $CD4^+$ T cells (Figure 5-7).

Figure 5-7; Correlation between the day 1 change in plasma CXCL10 and expression of CXCR3 by T cells



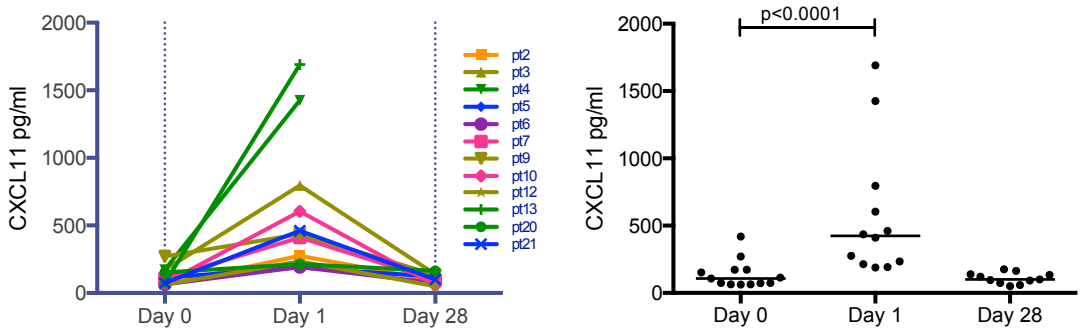

Left graph: $CD4^+$ T cells. Right graph: $CD8^+$ T cells. CXCR3 percentages represent the absolute change in CXCR3 expression by $CD4^+$ and $CD8^+$ T cells between Day 0 and Day 1. Change = Day 1 – Day 0 for each individual. R is calculated using Spearman coefficient for non-parametric data

## 5.5.2 CHANGES IN PLASMA CXCL11 DURING PEG-IFN/RIBAVIRIN THERAPY

### *Longitudinal changes in plasma CXCL11 concentration*

In addition to CXCL10, changes in plasma CXCL11 concentrations were assessed longitudinally during therapy in 12 patients. Similar to CXCL10, plasma CXCL11 increased significantly between day 0 and day 1 from a median of 106.8 to 423.0 pg/ml, and returned to baseline by day 28 ($p < 0.0001$) (Figure 5-8).

**Figure 5-8; Longitudinal plasma CXCL11 concentrations during PEG-IFN/ribavirin therapy**




Dotted vertical lines represent times of PEG-IFN dosing. Samples at these time-points were taken prior to PEG-IFN dosing. Left graph: coloured lines connect individual patient samples across time. Right graph: horizontal lines depict median values. Sufficient plasma was not available to analyse day 28 samples for 2 patients (pts 4 and 13).

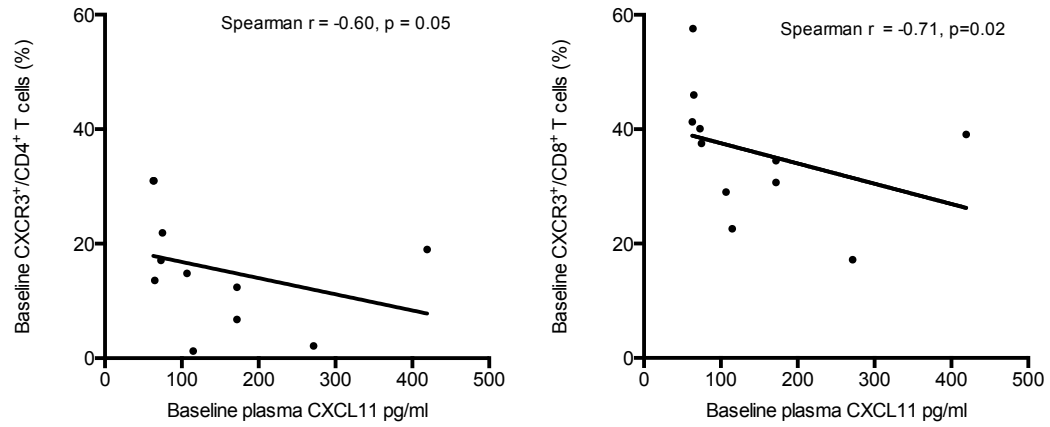
### *CXCL11 and circulating lymphocyte number*

There was no correlation between plasma CXCL11 and circulating lymphocyte counts at baseline, or after 1 day of PEG-IFN therapy.

### *CXCL11 and CXCR3 expression*

At baseline, there was a significant correlation between plasma CXCL11 and CXCR3 expression by circulating CD4+ and CD8+ T cells (Figure 5-9).

Figure 5-9; Correlation between plasma CXCL11 and CXCR3 expression by T cells at baseline




Left graph: $CD4^+$ T cells. Right graph: $CD8^+$ T cells. R is calculated using Spearman coefficient for non-parametric data.

Unlike the CXCL10 data, changes in CXCR3 expression by circulating T cells between day 0 and day 1 did not correlate with changes in plasma CXCL11 (Figure 5-10).

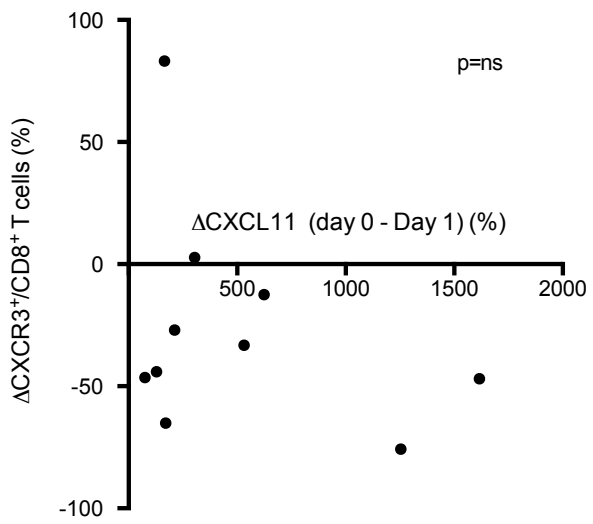


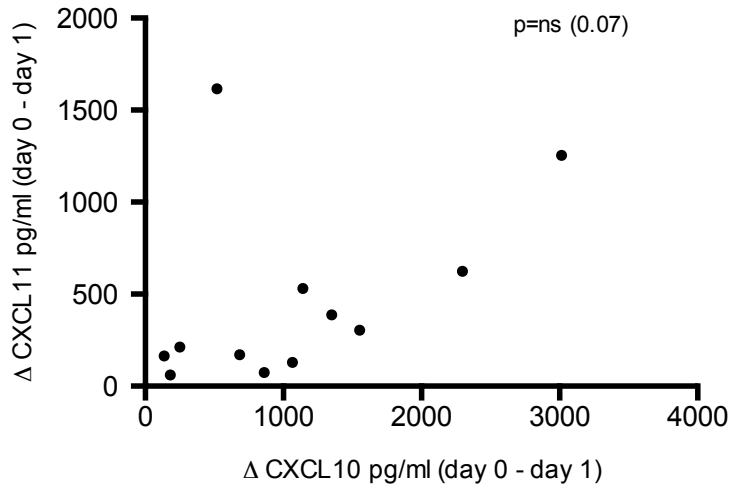

Figure 5-10; Relationship between day 1 changes in plasma CXCL10 and CXCR3 expression by CD8+ T cells

p = ns. CXCR3 percentages represent the absolute change in CXCR3 expression by $CD4^+$ and $CD8^+$ T cells between Day 0 and Day 1. (Change = day 1 – day 0 for each individual). R is calculated using Spearman coefficient for non-parametric data.

### 5.5.3 IS THERE A RELATIONSHIP BETWEEN CXCL11 AND CXCL10 INDUCTION?

Although there appeared to be positive correlation between day 1 changes in plasma CXCL10 and CXCL11, this was not statistically significant (p = 0.07) (Figure 5-11).

Figure 5-11; Change in plasma CXCL11 and CXCL10 between day 0 and day 1

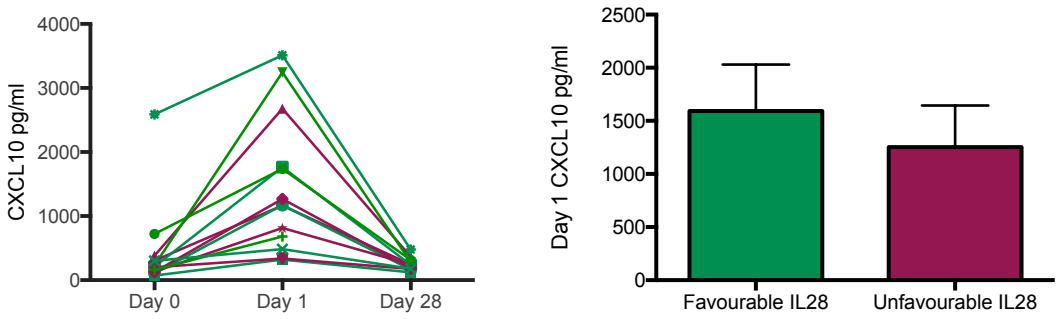



Change = day 1 – day 0 for each individual. R is calculated using Spearman coefficient for non-parametric data.

### 5.5.4 IS THERE A RELATIONSHIP BETWEEN CXCR3-BINDING CHEMOKINES AND HOST IL-28 STATUS?

Data suggests that in HCV infected individuals, IL-28 genotype effects interferon stimulated gene (ISG) expression – individuals with a favourable IL-28 genotype have lower hepatic ISG expression (Urban et al. 2010). It is therefore plausible that baseline circulating CXCL10 levels, as well as sensitivity to exogenous interferon-alpha (measured by CXCL10 induction), might relate to host IL-28 status. For these reasons, I next analysed the data set according to host IL-28 genotype. At baseline, and during therapy there was no relationship between plasma CXCL10 and host IL-28 genotype when patients were grouped according to favourable (T/T) or unfavourable IL-28 genotypes (T/G or G/G) (Figure 5-12).

Figure 5-12; Plasma CXCL10 concentration stratified by IL28 genotype




Favourable IL28 = green lines/bars. Unfavourable IL28 =purple lines/bars. The left graph represents longitudinal change across time in individual patients. The right graph compares day 1 plasma CXCL10 concentrations between groups.

## 5.6 KEY QUESTION 2: Is the liver a source of CXCR3-binding chemokines induced by IFN-α?

In the setting of liver disease, CXCR3-binding chemokines are known to be expressed by hepatocytes, cholangiocytes, and stellate cells in the liver (Curbishley et al. 2005). Having demonstrated that PEG-IFN causes a rapid increase in these chemokines in plasma, I next assessed whether, IFN-α directly induces production of these chemokines by hepatocytes.

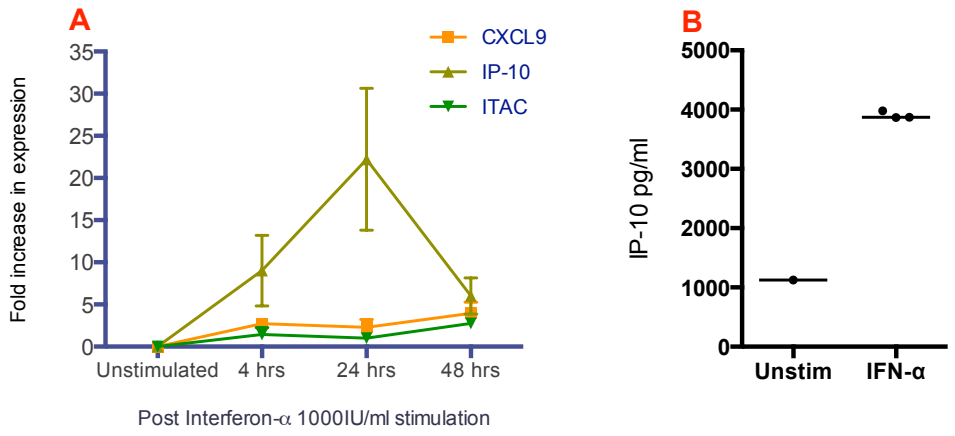
### 5.6.1 Huh7.5 mRNA EXPRESSION FOLLOWING IFN-α STIMULATION

Hepatoma cells (Huh7.5) cells significantly up-regulated mRNA expression of CXCL9, CXCL10 and CXCL11 as early as 4 hours after stimulation with 1000 IU/ml IFN-α (Figure 5-13-A).

### 5.6.2 CXCL10 PRODUCTION BY Huh7.5 CELLS FOLLOWING IFN-α STIMULATION

Following 24 hour stimulation with 1000 IU/ml IFN-α, Huh7.5 cell lysate contained 3906.9 pg/ml of CXCL10 (mean value of 3 separate samples measured by ELISA) (Figure 5-13-B)

Figure 5-13; CXCR3-binding chemokine production by Huh7.5 cells following IFN-α stimulation




Graph A: Huh 7.5 cells were stimulated with 1000 IU/ml for 4, 24 and 48 hours and mRNA was then extracted and quantified by qPCR (controlled using GAPDH reporter expression). Mean values of 3 separate samples ± SEM are plotted.
Graph B: CXCL10 production measured by ELISA of Huh 7.5 cell lysate – 3 samples stimulated with IFN-α for 24 hours are compared with a single unstimulated control. Unstim = unstimulated control

## 5.7 KEY QUESTION 3: Does IFN-α cause down-regulation of CXCR3 expression or induce death of CXCR3$^+$ lymphocytes?

Rather than inducing lymphocyte redistribution, the decrease in circulating CXCR3 expressing T cells during HCV therapy might alternatively be explained by IFN-α driven down-regulation of CXCR3 surface expression, or targeted death of this lymphocyte subset. These 2 possibilities were evaluated as described below. Three other chemokine receptors, CCR6, CCR9 and B7integrin were also evaluated in this set of experiments – this was to provide a comparison of changes with those of CXCR3, and also because these receptors were later evaluated in the mouse model of lymphocyte homing.

Example FACS plots of surface expression of the chemokine receptors CXCR3, CCR6, CCR9 and B7integrin by $CD8^+$ and $CD8^-$ T cells after 24 hours IFN-α are compared with untreated cells in Figure 5-14.

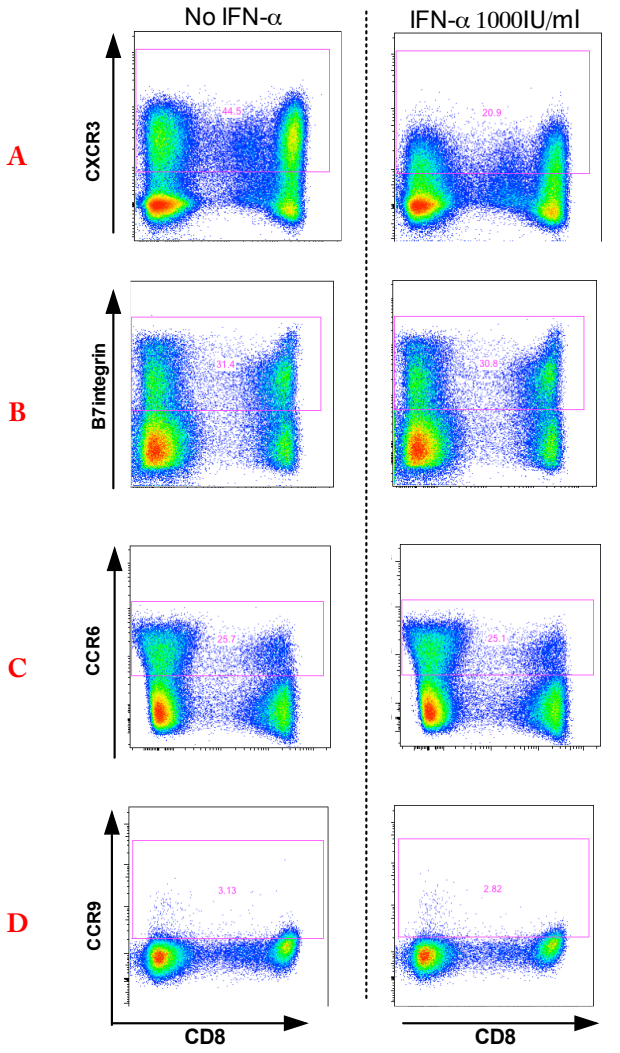
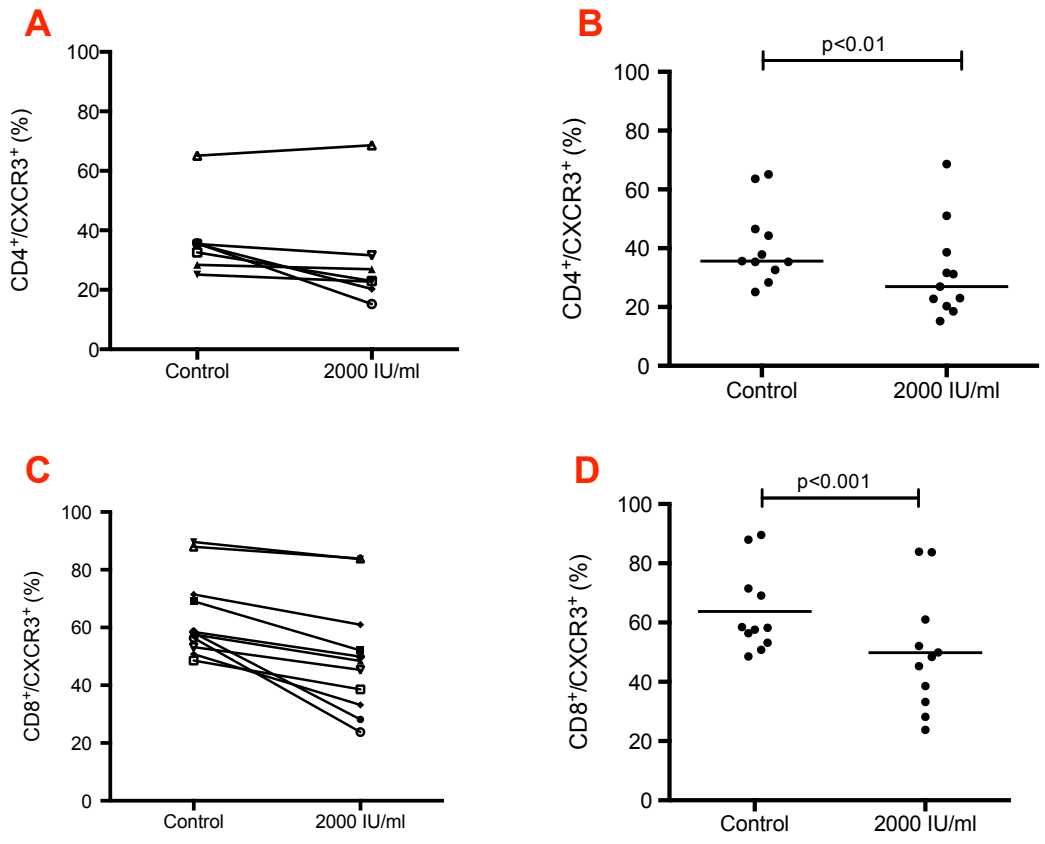



Figure 5-14; FACS plots comparing chemokine receptor expression between IFN stimulated and unstimulated CD3⁺ lymphocytes

Left column: control cells incubated in R10 for 24 hours. Right column: 1000 IU/ml IFN-α stimulated cells. Row (A): CXCR3 expression; Row (B): B7integrin expression; Row (C): CCR6 expression; and row (D): CCR9 expression by $CD8^+$ and $CD8^-$ T cells. These plots have been first gated on $CD3^+$, live PBMCs

After 24 hours stimulation in-vitro with IFN-α (2000 IU/ml), CD4⁺ and CD8⁺ T cells down-regulated surface expression of CXCR3; CD4⁺ T cells: 35.6% → 26.9%, $p < 0.01$, and CD8⁺ T cells: 58.2% → 48.4%, $p < 0.001$ (median of 7 healthy volunteer samples, p value: Wilcoxon signed rank test) (Figure 5-15).

Figure 5-15; Changes in CXCR3 expression by IFN-α stimulated T cells

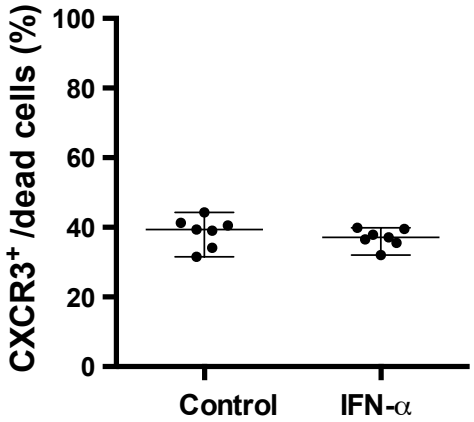



Plots represent samples from 7 individual healthy volunteers. Top row (A&B): $CD4^+$ T cells. Bottom row (C&D): $CD8^+$ T cells. Left column: demonstrates the changes for paired samples (joined by lines) from individual volunteers. Right column: horizontal lines represent group medians. Cells were stimulated at IFN-α concentrations of 0 IU/ml (control), and 2000 IU/ml for 24 hours.

In contrast to CXCR3, surface expression of chemokine receptors CCR6, CCR9 and B7integrin did not change when cultured in the presence of IFN-α (Appendix 17).

Next, the possibility of IFN-α induced cell death specific for the $CXCR3^+$ T cell population was investigated. Dead cells were defined using live/dead staining (Invitrogen L-10119). After 24 hours IFN-α stimulation, there was no increase in the proportion of dead cells expressing CXCR3 making this an unlikely explanation for the observed changes in CXCR3 expression by lymphocytes in vivo (Figure 5-16).

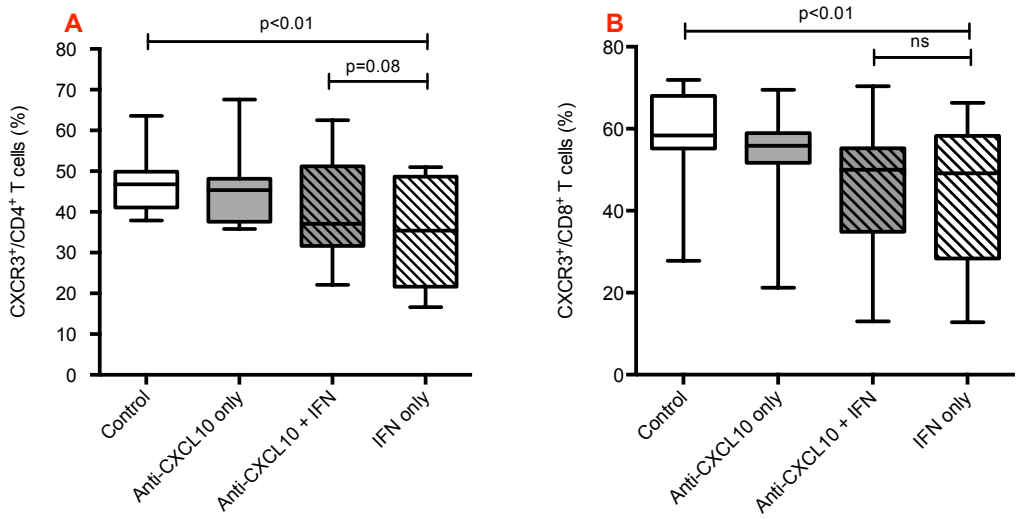
Figure 5-16; Changes in CXCR3+ expression by dead PBMCs after in-vitro culture with IFN-α




Cells were stimulated with IFN-α at concentrations of 0 IU/ml (control), and 2000 IU/ml for 24 hours. Plots represent samples from 7 individual healthy volunteers. Horizontal lines depict group medians. The represented cell population is first gated on single cells/PBMCs followed by dead cells. Dead cells = cells positive for live/dead staining (Invitrogen LIVE/DEAD stain) – this reactive dye permeates compromised membranes of necrotic cells and reacts with free amines both in the interior and on the cell surface resulting in intense fluorescent staining.

There is evidence that ligand binding causes down-regulation of CXCR3 surface expression (Meiser et al. 2008; Sauty et al. 2001). To investigate the possibility that IFN-α caused CXCR3 down-regulation indirectly through the induction of CXCL10, PBMCs from 8 healthy volunteers were next incubated with IFN-α in the presence of CXCL10 blocking antibody (Figure 5-17). There is a suggestion that blockade of CXCL10 abrogated down-regulation of CXCR3 ($p = 0.08$ for $CD4^+$ T cells). It is possible that induction of the other CXCR3-binding chemokines (CXCL11 and CXCL9) by IFN-α might similarly contribute to CXCR3 down-regulation.

Figure 5-17; In-vitro changes in CXCR3 expression by lymphocytes following IFN-α stimulation in the presence of CXCL10 blockade




Left graph (A): $CD4^+$ T cells. Right graph (B): $CD8^+$ T cells. Cells were incubated for 24 hours before analysis in media alone (white box), with 5 µg/ml CXCL10 blocking antibody alone (grey box), blocking antibody together with 1000 IU/ml IFN-α (grey box with diagonal lines), or IFN-α alone (white box with diagonal lines). Data represent results using PBMCs from 8 healthy individuals.

## 5.8 KEY QUESTION 4: Does exogenous IFN-α drive enrichment of hepatic CXCR3$^+$ lymphocytes in a mouse model?

Despite having shown that; i) IFN-α induces CXCR3-binding chemokines in-vivo, and ii) the liver is one potential source of these chemokines, the finding that IFN-α also causes down-regulation of CXCR3 raised the possibility that this alone might explain the decrease in circulating CXCR3$^+$ lymphocytes observed during PEG-IFN/ribavirin therapy in the THIRST trial (Chapter 4). A murine model was therefore employed to investigate whether exogenous IFN-α drives hepatic enrichment of CXCR3$^+$ lymphocytes.

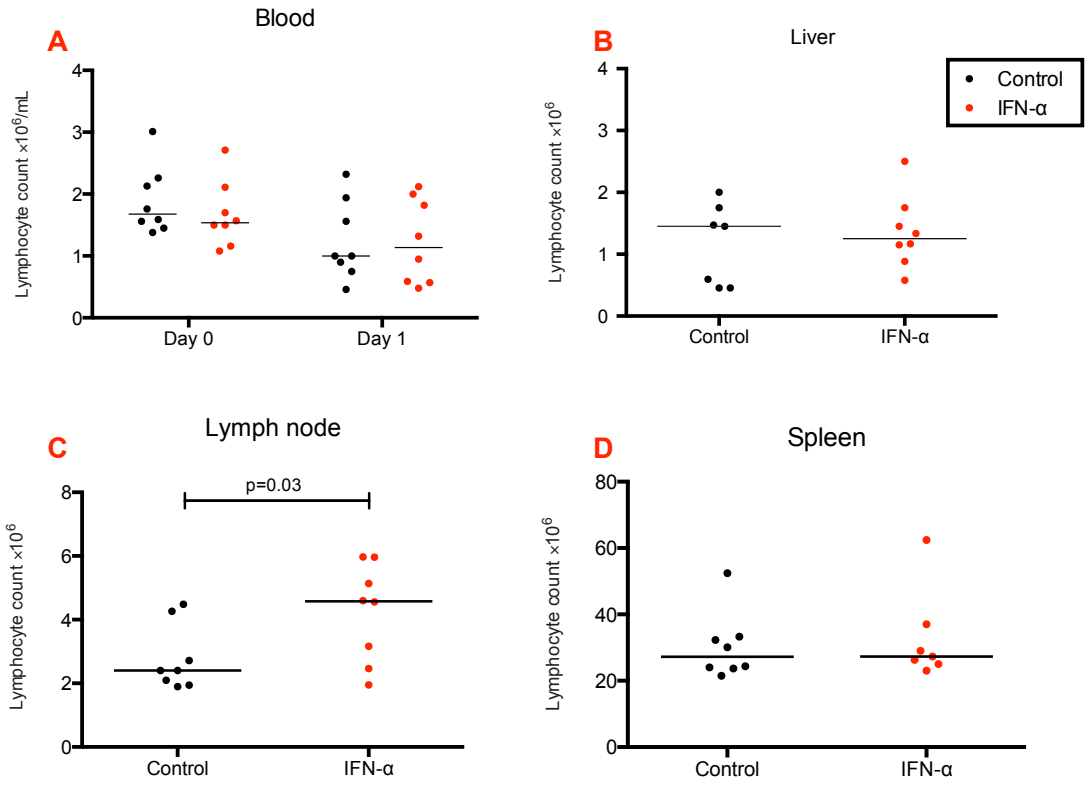
For experiment/procedural detail please see Methods 2.13, page 96. In brief, 100,000 IU of IFN-α was administered subcutaneously to 8 C57BL/6 mice and subsequent changes in the number and homing phenotype of circulating and organ sequestered lymphocytes were compared with 8 control mice.

In addition to CXCR3 expression, the chemokine receptors CCR6, CCR9 and B7integrin were evaluated in this experiment. The role of CCR6 as a hepatic chemokine receptor is discussed in Section 4.8 (page 178). CCR9 and α4/B7integrin are gut homing receptors and were therefore chosen for comparative analysis. CCR9 binds the chemokine CCL25 which, outside of the thymus, is almost exclusively expressed by epithelial cells in the crypts of the small intestine (Kunkel et al. 2000; Papadakis et al. 2000). Integrins on the other hand, are major mediators of cell adhesion. A4/β7integrin directs trafficking of naïve and memory T cells to intestinal sites of inflammation via interactions with mucosal addressin cell adhesion molecule (MAdCAM-1) (Erle et al. 1994).

### 5.8.1 DO CIRCULATING OR ORGAN SEQUESTERED LYMPHOCYTE NUMBERS CHANGE FOLLOWING ADMINISTRATION OF IFN-α IN MICE?

One day after IFN-α administration, there was no difference in the number of circulating, liver or splenic lymphocytes between groups (IFN-α vs. control group). However, greater numbers of lymphocytes were isolated from the pre-auricular lymph nodes of IFN-α treated mice ($4.6 \times 10^6$ vs. $2.4 \times 10^6$ cells, median values, p = 0.03) (Figure 5-18).

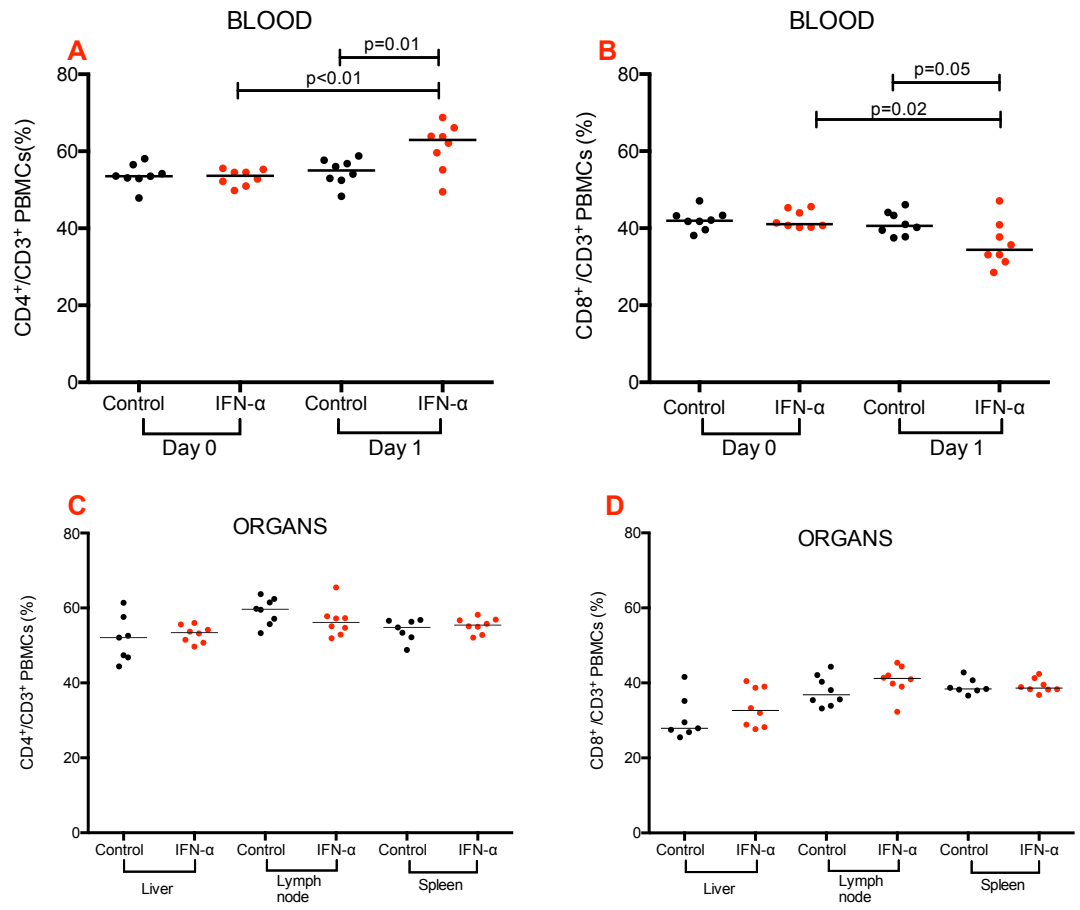
Figure 5-18; Circulating and organ sequestered lymphocyte counts – IFN-α and control groups compared




Lymphocytes were quantified in the blood (A), liver (B), pre-auricular lymph nodes (C) and spleens (D) of mice, 24 hours after administering 100,000 IU/ml IFN-α (red points) for comparison with controls (black points). Additionally, circulating lymphocyte counts were obtained prior to IFN-α administration on day 0 for comparison with day 1 samples (A). The lymph node quantity is the combined number of cells isolated from both pre-auricular lymph nodes. Horizontal lines depict group medians.

Despite there being no change in the total number of circulating lymphocytes following IFN-α administration, as proportions of $CD3^+$ cells, like they had in humans (see page 155), circulating $CD8^+$ T cells decreased from a median of 41.1% to 34.4% (p = 0.02) (Figure 5-19-A&B). This suggests that $CD8^+$ T cells preferentially migrate from circulation. Although there was a suggestion that these cells were enriched in the liver and lymph nodes of IFN-α treated mice, this was not significant (p = 0.11 and 0.22 respectively) (Figure 5-19-D).

Figure 5-19; Proportions of circulating, and organ sequestered lymphocytes comprised of $CD4^+$ T cells and $CD8^+$ T cells – IFN-α and control groups compared

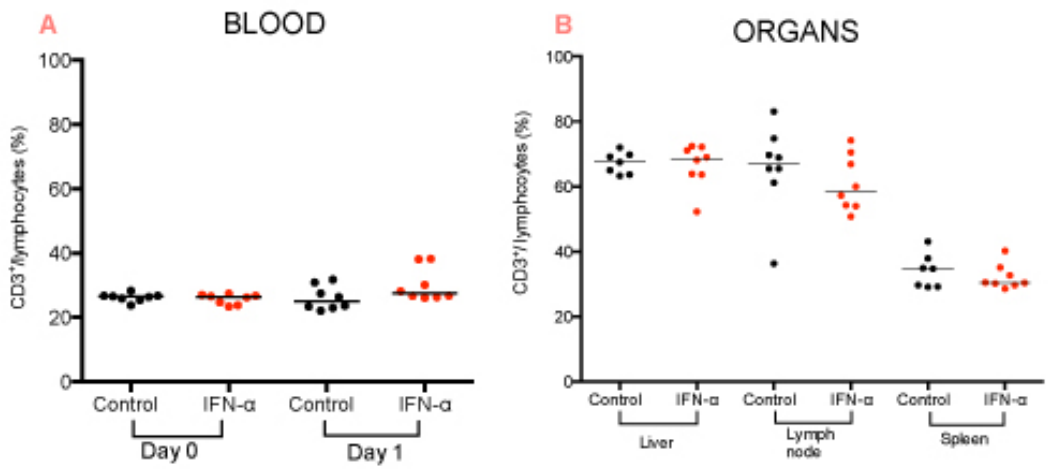



IFN group (red) compared with control group (black). Horizontal lines represent median values. The left column (A&C) represents $CD4^+$ T cells and the right column (C&D) represents $CD8^+$ T cells. The top row is blood samples across day 0 and day 1 groups. The bottom row represents organ sequestered T cells as indicated.

One explanation for the failure to see an enrichment of either $CD4^+$ or $CD8^+$ T cells in pre-auricular lymph nodes despite an increase in absolute cell number is this increase was caused by sequestration of $CD3^-$ lymphocytes. For this reason, the relative differences of $CD3^+$ and $CD3^-$ populations in blood and organs were next evaluated.

Despite a suggestion that the $CD3^-$ population was enriched in lymph nodes of IFN-α treated mice this was not significant ($p = 0.26$) (Figure 5-20-B).

Figure 5-20; $CD3^+$ lymphocytes as proportions of total lymphocytes isolated from blood and organs – IFN-α and control groups compared




IFN-α group (red) and control group (black). The left graph depicts $CD3^+$ lymphocytes in blood samples from Day 0 (prior to IFN-α) for both groups compared and day 1 samples. The right graph depicts $CD3^+$ lymphocytes isolated from organs. Horizontal lines represent group medians.

### 5.8.2 DOES CXCR3 EXPRESSION ON MURINE LYMPHOCYTES CHANGE FOLLOWING ADMINISTRATION OF IFN-α?

Example FACS plots of CXCR3 expression by $CD8^+$ T cells isolated from 2 mice (1 untreated control, and 1 treated with IFN-α) are demonstrated in Figure 5-21. In untreated mice (left column of FACS plots), hepatic T cells more frequently express CXCR3 than those isolated from blood, lymph node and spleen (CD8; 25.9% vs. 19.8%, 16.2% and 25.2% respectively, CD4; 36.6% vs. 4.4%, 3.4% and 10.8% respectively, median values, all $p<0.05$).

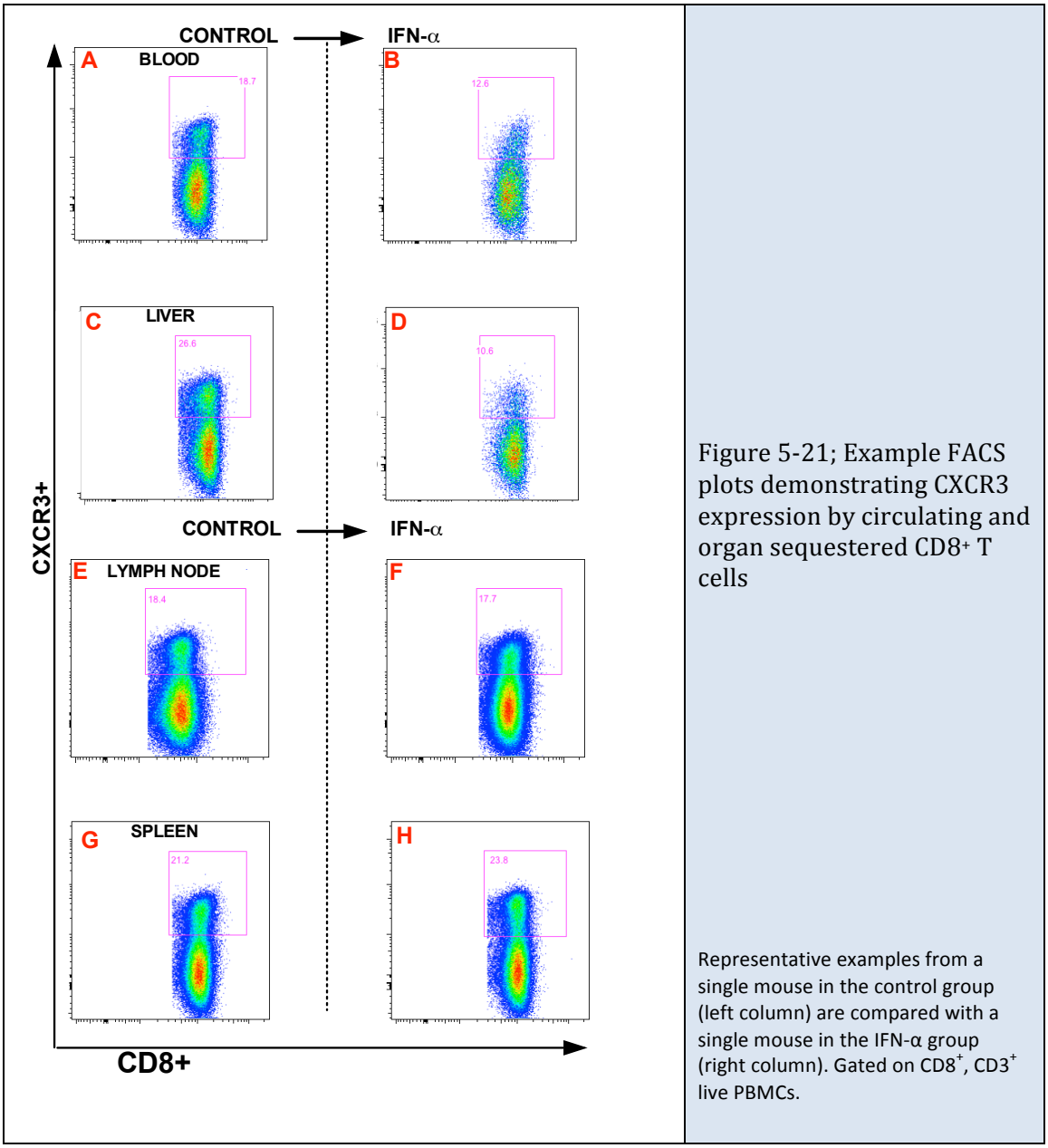


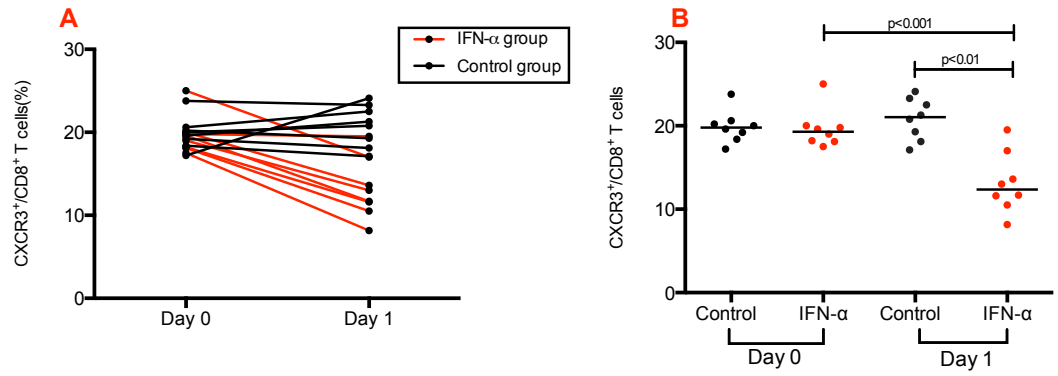

Figure 5-21; Example FACS plots demonstrating CXCR3 expression by circulating and organ sequestered $CD8^+$ T cells

Representative examples from a single mouse in the control group (left column) are compared with a single mouse in the IFN-α group (right column). Gated on $CD8^+$, $CD3^+$ live PBMCs.

### *CXCR3 expression by murine CD8⁺ T cells*

One day after administering IFN-α, CXCR3⁺ expression by CD8⁺ T cells decreased by a median of 6.9% ($p < 0.01$) (Table 5-1 and Figure 5-22).

Figure 5-22; CXCR3 expression by circulating murine CD8⁺ T cells – IFN-α and control groups compared




IFN-α group (red) and control group (black). The left graph depicts changes across time in individual mice (joined by lines). The right graph depicts individual values at each time-point arranged in columns according to group and day of collection. Horizontal lines represent median values. P values for differences within groups are calculated using Wilcoxon signed rank test while p values across groups are calculated using the Mann-Whitney U test for unpaired data.

Similarly, CD8⁺ T cells isolated from the liver and spleens (but not lymph nodes) of mice in the IFN-α group expressed less CXCR3⁺ than those of control mice (Figure 5-23)

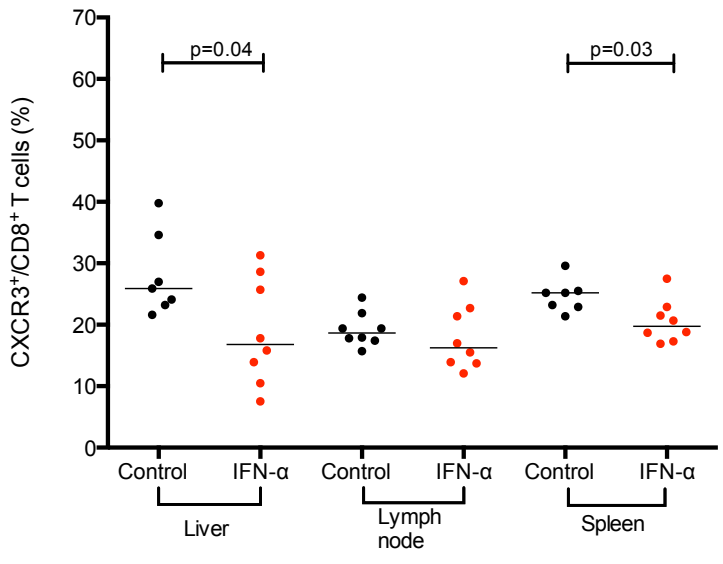


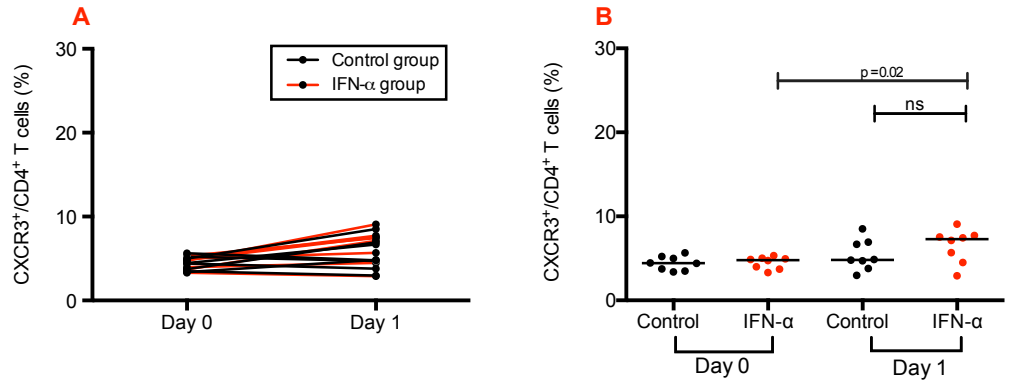

Figure 5-23; CXCR3 expression by murine CD8⁺ T cells isolated from liver, pre-auricular lymph nodes and spleen

IFN group (red) compared with control group (black). Horizontal lines depict group medians.

### *CXCR3 expression by murine CD4⁺ T cells*

Unlike CD8⁺ T cells, there was no change in CXCR3 expression by CD4⁺ T cells following administration of IFN-α (Figure 5-24 and Table 5-1).

Figure 5-24; CXCR3 expression by circulating CD4⁺ T cells in mice
IFN-α and control groups compared




IFN-α group (red) and control group (black). Left graph: changes across time (individual mice joined by lines). Right graph: individual values at each time-point arranged in columns according to group and day of collection. Horizontal lines represent median values. P values for differences within groups are calculated using Wilcoxon signed rank test, while p values across groups are calculated using the Mann-Whitney U test for unpaired data.

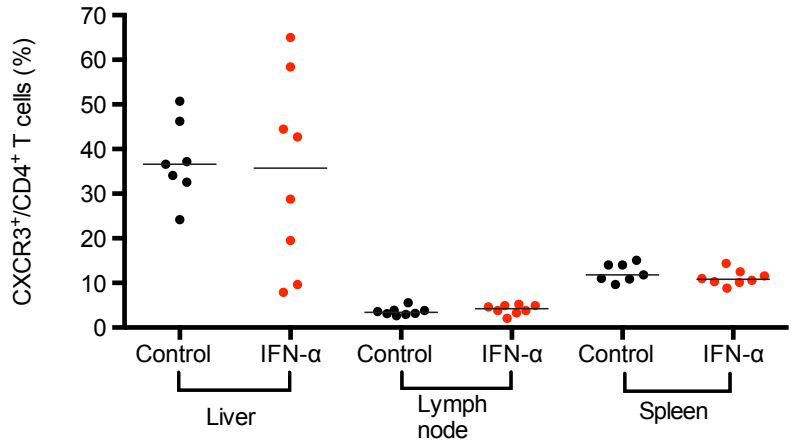
Table 5-1; Percentages of CD4⁺ and CD8⁺ T cells expressing CXCR3

| | **Control group** | **IFN-α group (day 0)** | **IFN-α group (day 1)** | **P value** |
|---|---|---|---|---|
| CXCR3/CD8⁺ T cells | | | | |
| Blood | 21.1% (Day1) | 19.3% | 12.4% | p < 0.001 (Control vs. IFN-α)<br>p < 0.01 (IFN-α: day 0 vs. day 1) |
| Spleen | 25.2% | | 19.8% | p = 0.03 |
| Liver | 25.9% | | 16.8% | p = 0.04 |
| Lymph nodes | 18.7% | | 16.3% | ns |
| CXCR3/CD4⁺ T cells | | | | |
| Blood | 4.8% (Day 1) | 4.8% | 7.3% | p = 0.27 (Control vs. IFN-α)<br>p = 0.02 ( IFN-α: day 0 vs. day 1) |
| Spleen | 11.8% | | 10.8% | ns (p = 0.30) |
| Liver | 36.6% | | 35.8% | ns (p = 0.78) |
| Lymph nodes | 3.4% | | 4.2% | ns (p = 0.23) |

Cells isolated from blood, liver, lymph node and spleens of mice in the IFN and control groups on day 1. For blood analysis – the IFN group on day 1 is compared with both the Day 0 IFN group samples and also the Day 1 control group samples – p values for Day 0 vs. Day 1 blood are paired analyses calculated using Wilcoxon matched pairs signed rank test.

In addition, comparing the IFN group with control mice, there was no difference in $CXCR3^+$ expression by $CD4^+$ T cells isolated from any organ (Figure 5-25).

Figure 5-25; CXCR3 expression by $CD4^+$ and $CD8^+$ T cells isolated from mouse organs. IFN-α and control groups compared

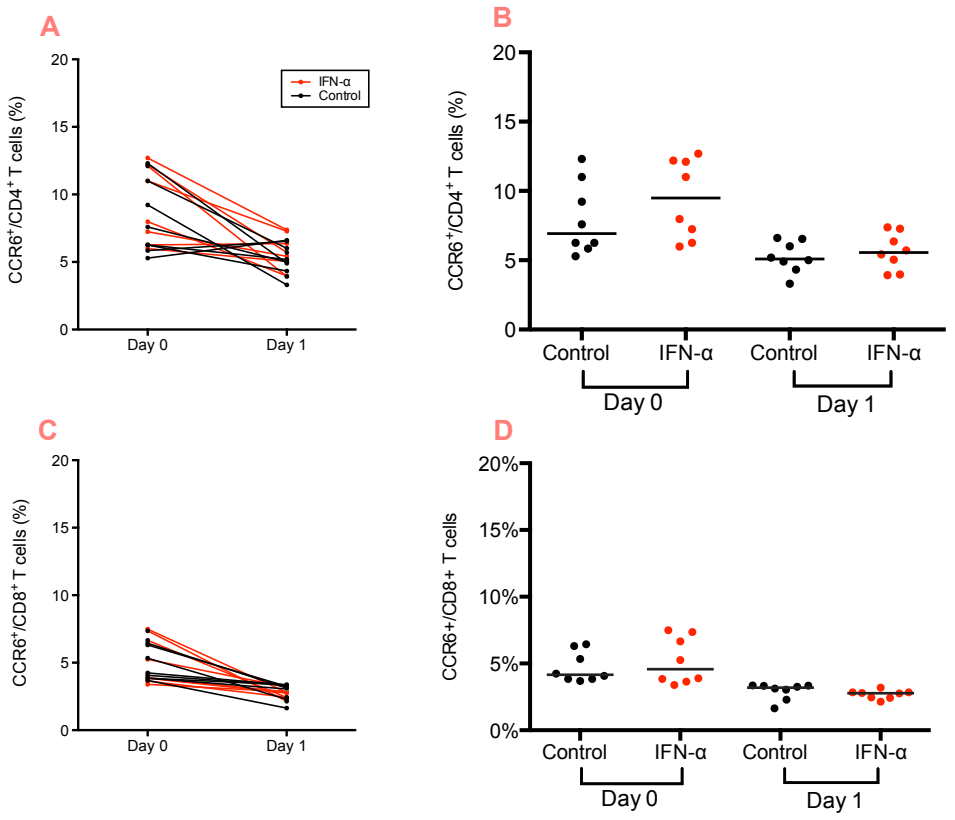



IFN-α group (red) compared with controls (black). Horizontal lines represent group medians. Values are arranged in columns according to group and organ.

### *CCR6 expression by murine T cells*

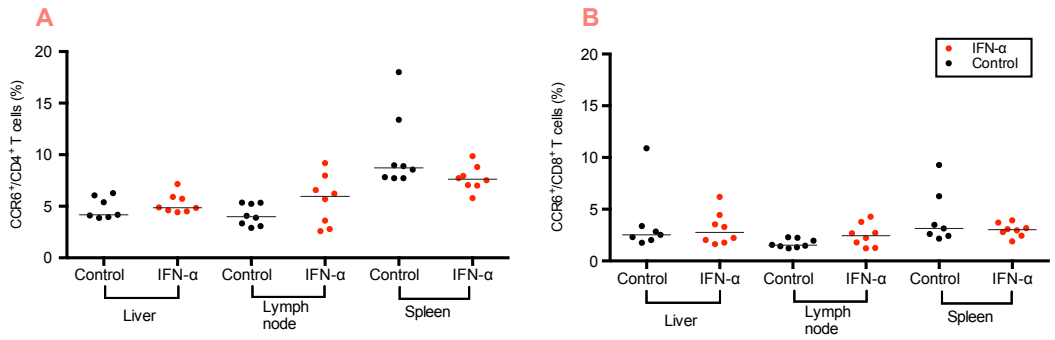
Although CCR6 expression by circulating CD4+ and CD8+ T cells decreased in IFN-α treated mice between day 0 and day 1, this also occurred in the control group – suggesting a separate factor, unrelated to IFN-α had influenced this change (Figure 5-26). Furthermore, there was no difference in CCR6 expression by CD4+ or CD8+ T cells isolated from the corresponding organs of each group (Figure 5-27).

Figure 5-26; CCR6 expression by circulating murine CD8+ T cells IFN-α and control groups compared




IFN-α group (red) and control group (black). Top row (A&B): CD4+ T cells. Bottom row (C&D): CD8+ T cells. Left column: changes across time in individual mice (joined by lines). Right column: individual values at each time-point arranged in columns according to group and day. Horizontal lines represent group medians.

Figure 5-27; CCR6 expression by lymphocytes isolated from mouse organs – IFN-α and control groups compared




IFN-α group (red) compared with controls (black). Left graph: $CD4^+$ T cells. Right graph: $CD8^+$ T cells. Individual values at each time-point are arranged in columns according to group and organ. Horizontal lines represent median values.

### *CCR9 expression by murine T cells*

An example of CCR9 staining on $CD8^+$ and $CD8^-$ T cells isolated from murine liver is given in Appendix 18.

At baseline, greater proportions of circulating murine $CD4^+$ and $CD8^+$ T cells express CCR9 than their human counterparts; $CD4^+$: 2.6% vs. 1.6% ($p=0.02$) and $CD8^+$: 24.0% vs. 3.8% respectively, ($p<0.01$). Human data is based on 7 healthy adult samples.

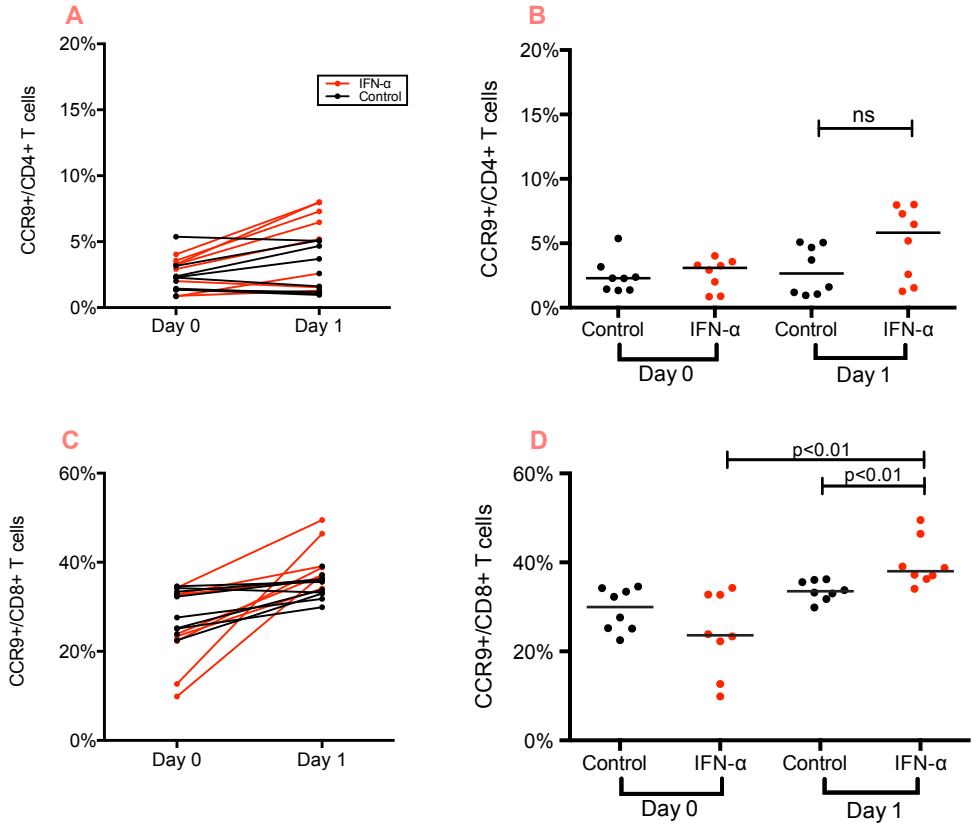
Table 5-2; CCR9 expression by $CD8^+$ and $CD4^+$ T cells

| | **Control group** | **IFN-α group (day 0)** | **IFN-α group (day 1)** | **P value** |
|---|---|---|---|---|
| CCR9/$CD8^+$ T cells | | | | |
| Blood | 33.5% (day 1) | 23.7% | 38.0% | $p < 0.01$ (Control vs. IFN-α)<br>$p < 0.01$ (IFN-α: day 0 vs. day 1) |
| Spleen | 35.2% | | 38.7% | ns ($p = 0.19$) |
| Liver | 31.1% | | 36.6% | ns |
| Lymph nodes | 26.4% | | 32.6% | ns |
| CCR9/$CD4^+$ T cells | | | | |
| Blood | 2.6% (day 1) | | 5.0% | ns |
| Spleen | 7.6% | | 9.0% | ns ($p = 0.28$) |
| Liver | 5.7% | | 9.4% | ns ($p = 0.53$) |
| Lymph nodes | 3.5% | | 3.5% | ns ($p$ 0.44) |

Cells isolated from blood, liver, lymph node and spleens of mice in the IFN and control groups on day 1. For blood analysis – the IFN- α group on day 1 is compared with both the Day 0 IFN-α group samples and also the day 1 control group samples. P values for Day 0 vs. Day 1 blood are paired analyses calculated using Wilcoxon matched pairs signed rank test.

Between day 0 and day 1, CCR9 expression by circulating $CD8^+$ T cells increased in mice receiving IFN-α from a median of 23.7% to 38.0% ($p<0.01$) but remained static on $CD4^+$ T cells (Table 5-2, and Figure 5-29).

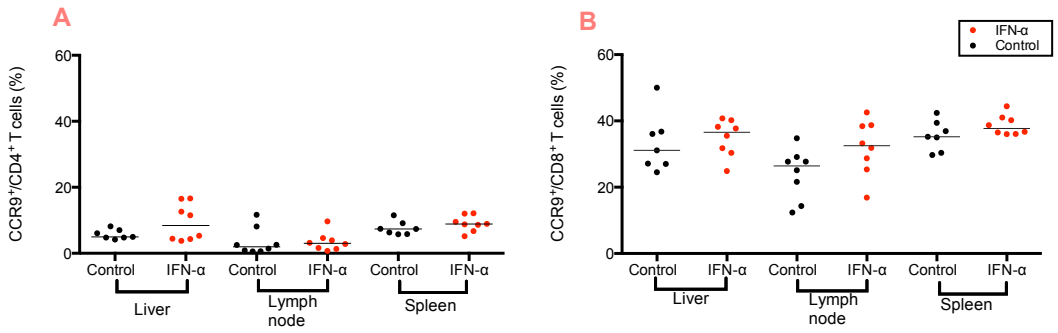
Figure 5-28; CCR9 expression by circulating murine CD8⁺ T cells – IFN-α and control groups compared




IFN-α group (red) and control group (black) compared. Top row (A&B): $CD4^+$ T cells. Bottom row (C&D): $CD8^+$ T cells. Left column: changes across time in individual mice (joined by lines). Right column: individual values at each time-point arranged in columns according to group and day. P values for differences within groups are calculated using Wilcoxon signed rank test, while p values across groups are calculated using the Mann-Whitney U test for unpaired data. Horizontal lines represent median values.

Although CCR9 expression by both $CD4^+$ and $CD8^+$ lymphocytes appeared to be greater in organs from the IFN-α group, these differences were not significant (Figure 5-29).

Figure 5-29; CCR9 expression by lymphocytes isolated from mouse organs – IFN-α and control groups compared

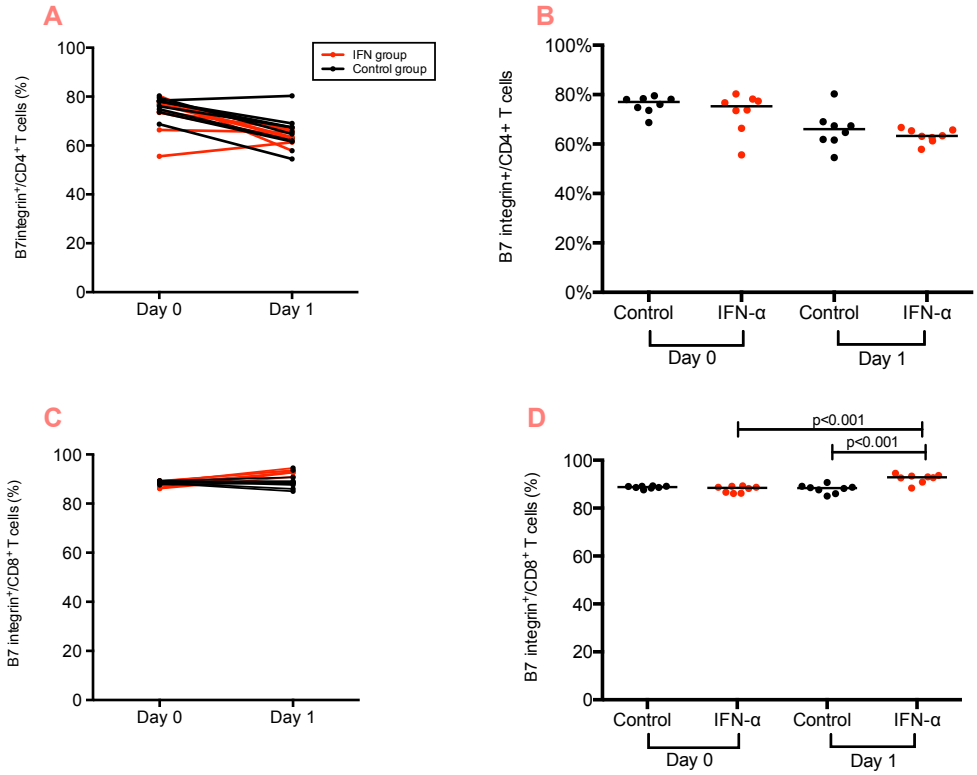



IFN-α group (red) and control group (black) compared. The left graph (A) represents $CD4^+$ T cells and the right graph (B) represents $CD8^+$ T cells. Individual values are arranged in columns according to group and organ. Horizontal lines represent median values.

### *B7 integrin expression by murine T cells*

Circulating CD8+ T cells expressing B7integrin increased significantly between day 0 and day 1 in mice receiving IFN-α, while no change was observed for CD4+ T cells (Figure 5-30).

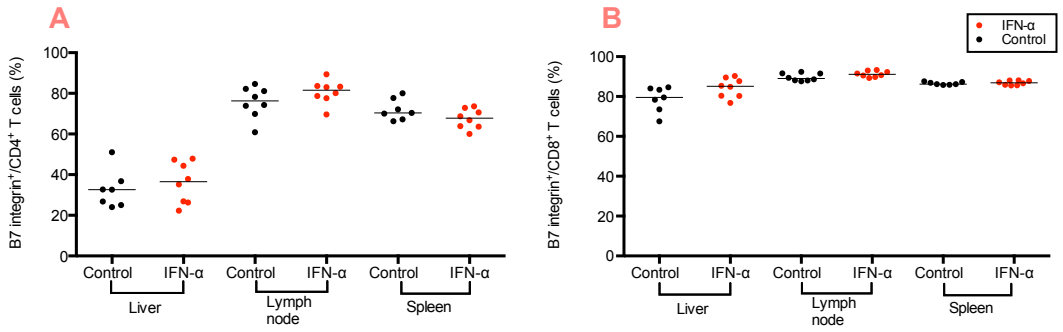
Figure 5-30; B7integrin expression on circulating murine CD8+ T cells – IFN-α and control groups compared




CD4+ (Top row; A&B) and CD8+ T cells (Bottom row; C&D). IFN-α group (red) and control group (black) compared. Horizontal lines represent median values. Graphs in the left column depict changes across time in individual mice (joined by lines). Graphs in the right column depict individual values at each time-point arranged in columns according to group and day. P values for differences within groups are calculated using Wilcoxon signed rank test, while p values across groups are calculated using the Mann-Whitney U test for unpaired data.

There was no significant difference between the IFN-α and control group in B7integrin expression by CD4+ or CD8+ T cells isolated from any organ (Figure 5-31).

Figure 5-31; B7integrin expression by T cells extracted from mouse organs – IFN-α and control groups compared



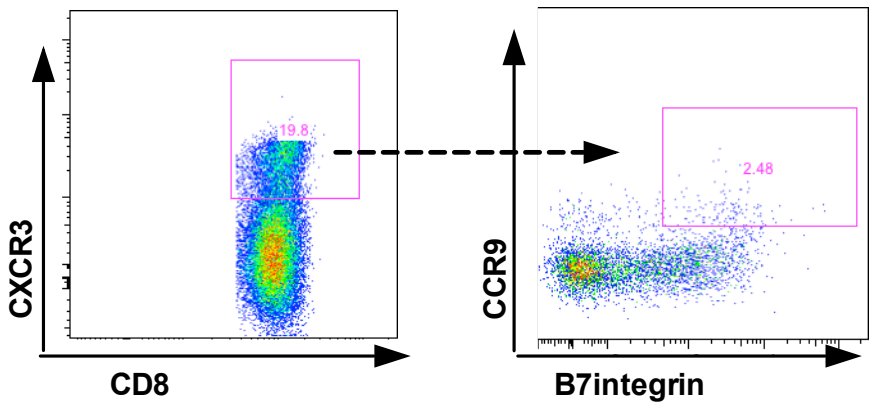

IFN group (red) and control group (black) compared. The left graph (A) represents $CD4^+$ T cells and the right graph (B) represents $CD8^+$ T cells. Individual values at each time-point are arranged in columns according to group and organ. Horizontal lines represent group median.

### *B7integrin/CCR9 negative cells*

Based on the primary hypothesis of this chapter, the $CXCR3^+$ T cell population was expected to be enriched in the liver of IFN-α treated mice – my finding to the contrary might theoretically be explained by masking of this enrichment as a result of IFN-α driven CXCR3 down-regulation. Therefore, I next evaluated co-expression of chemokine receptors to establish whether differential expression of gut homing receptors (CCR9 and B7integrin) could be used as an alternative way of defining the $CXCR3^+$ cell population, thereby overcoming the confounding factor of CXCR3 down-regulation.

I found that in mice, circulating $CXCR3^+$ $CD8^+$ T cells are rarely positive for CCR9 or B7integrin – only a median of 2.6% of $CXCR3^+$ $CD8^+$ T cells co-express B7integrin and CCR9, as demonstrated in the example below (Figure 5-32).

Figure 5-32; FACS plots demonstrating CCR9/B7integrin expression on CXCR3$^+$ CD8$^+$ T cells




Gated on CXCR3$^+$ CD8$^+$ T cells – these cells only rarely co-express CCR9 and B7integrin as is demonstrated by the right FACS plot

Furthermore, this analysis demonstrated that:

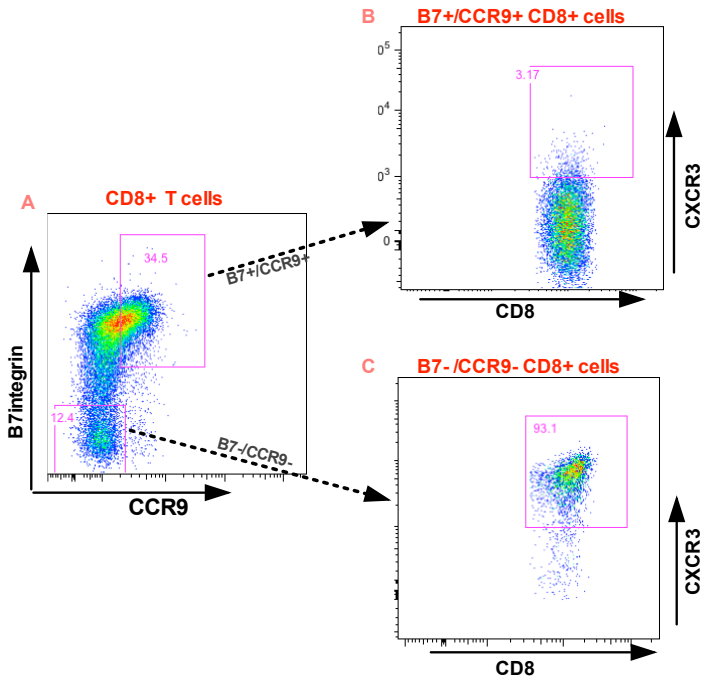
i. Circulating B7integrin$^+$/CCR9$^+$ CD8$^+$ T cells only rarely express CXCR3 (Figure 5-33-B)

and,

ii. Circulating B7integrin$^-$/CCR9$^-$ CD8$^+$ T cells almost universally express CXCR3 (Figure 5-33-C)

Combining these findings, we can conclude that B7integrin$^+$/CCR9$^+$ CD8$^+$ T cells and CXCR3$^+$ CD8$^+$ T cells are 2 distinct cell populations. Furthermore, considering CCR9$^-$/B7$^-$ CD8$^+$ T cells almost universally express CXCR3, and IFN-α does not alter expression of these chemokine receptors, this population can be used as a surrogate for CXCR3$^+$ CD8$^+$ T cells.

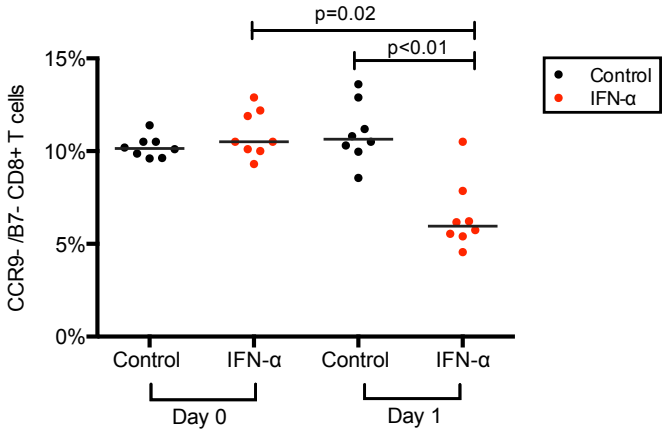
Figure 5-33; CXCR3 expression on CCR9⁺/B7⁺ CD8⁺ T cell subsets




(A) First gated on $CD8^+$ T cells. CXCR3 expression on $B7^+/CCR9^+$ subpopulation (B) and on $B7^-/CCR9^-$ subpopulation (C)

In keeping with this conclusion, as expected (and in keeping with changes observed in circulating CXCR3⁺ cells) – circulating $CCR9^-/B7^-$ $CD8^+$ T cells decrease after administration of IFN-α (Figure 5-34).

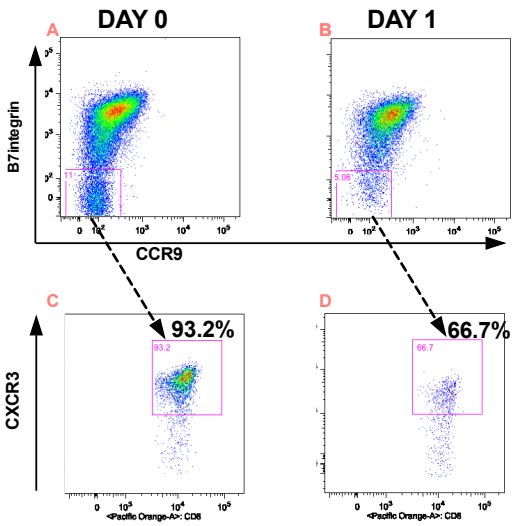
Figure 5-34; The change in circulating CCR9⁻/B7integrin⁻ CD8⁺ T cells – IFN-α and control groups compared




IFN-α group (red) and control group (black). Individual values are arranged in columns according to group and day. P values for differences within groups are calculated using Wilcoxon signed rank test, while p values across groups are calculated using the Mann-Whitney U test for unpaired data. Horizontal lines represent group medians.

An example of this change in an individual mouse treated with IFN-α is demonstrated by FACS plots in Figure 5-35.

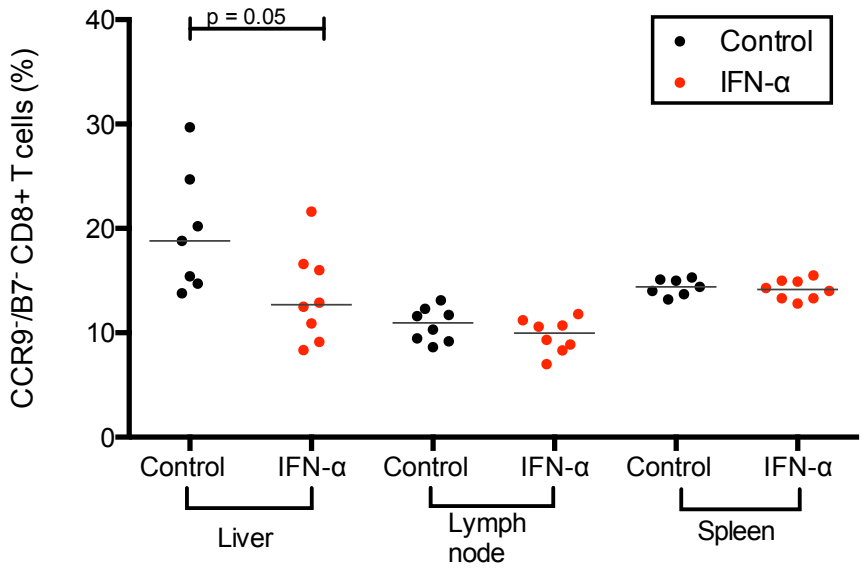
Figure 5-35 FACS plots demonstrating the decrease in B7integrin⁻/CCR9⁻ CD8⁺ T cells (together with decreases in CXCR3 expression) across time in an individual mouse receiving IFN-α




CCR9⁻/B7⁻ cells (blocked in pink in the top left and top right plots) decrease proportionally as does the percentage of this population expressing CXCR3 (squared in pink in the bottom 2 plots).

Using CCR9$^-$/B7$^-$ CD8$^+$ T cells as surrogates for the CXCR3$^+$ population, hepatic enrichment of this population in the IFN-α group was expected. This was, however, not the case – in fact, CCR9$^-$/B7$^-$ CD8$^+$ T cells were less common in the liver of treated mice than controls (Figure 5-36). There was, however, no difference between groups in the relative proportions of this population in the lymph nodes or spleen (Figure 5-36).

Figure 5-36 Organ sequestered CCR9$^-$/B7integrin$^-$ CD8$^+$ T cells – IFN-α and control groups compared

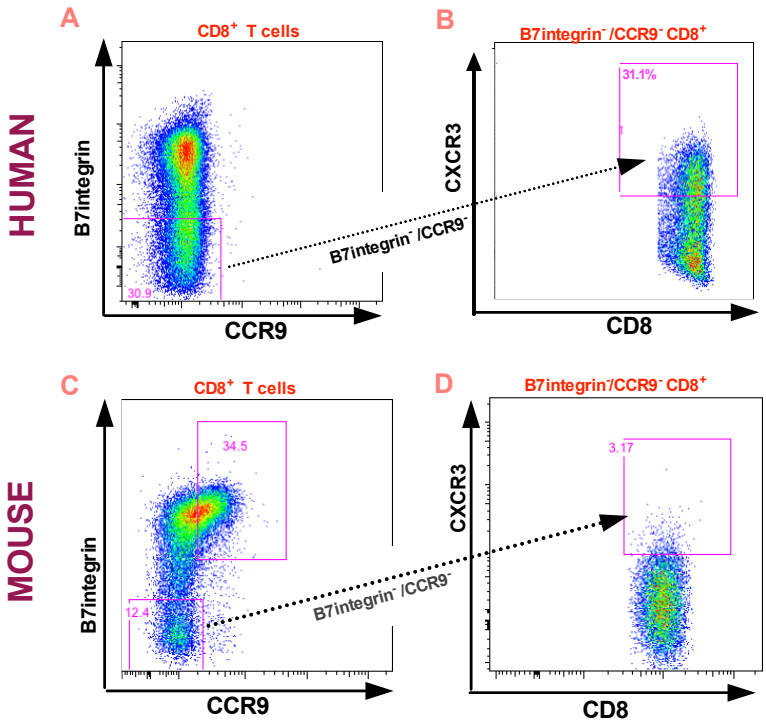



IFN-α group (red) compared with control group (black). Individual values are arranged in columns according to group and organ. Horizontal lines represent group medians.

### *CCR9⁻, B7⁻ CD8⁺ T cells; mice vs. humans*

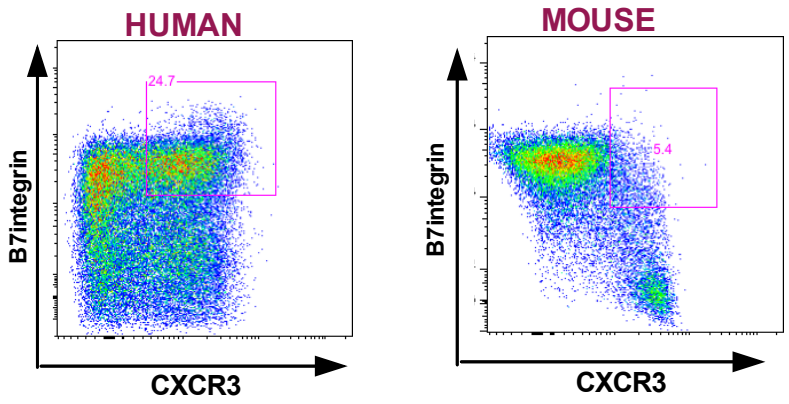
I next evaluated whether the same distinction between CCR9⁺/B7⁺ and CXCR3⁺ CD8⁺ T cells could be made for humans. In contrast to the murine data, based on chemokine receptor co-expression, there was significant overlap between these 2 subsets in humans (Figure 5-37 and Figure 5-38).

Figure 5-37; B7integrin⁻, CCR9⁻ CD8⁺ T cell populations and their respective CXCR3 expression – mice and humans compared




The top row is representative plots from human PBMCs. The bottom row represents equivalent figures from a representative murine sample. The left column plots are first gated on PBMCs/live/CD3⁺ cells.
The right column is gated on respective B7integrin⁻/CCR9⁻ cell populations.

Figure 5-38; FACS plots demonstrating CXCR3 expression on B7integrin$^+$ CD8$^+$ T cells in humans and mice




The left figure is a representative plot from a human sample of PBMCs –this demonstrates that a significant proportion of B7integrin$^+$ cells are also CXCR3$^+$. The right figure is the equivalent figure from a representative murine sample.

## 5.8.3 SUMMARY OF MURINE EXPERIMENTAL RESULTS

Table 5-3; Summary of in-vivo IFN-α induced changes in chemokine receptor expression by murine lymphocytes

| Chemokine | Cell type | Change |
|---|---|---|
| CXCR3 | CD4$^+$ | No change |
| | CD8$^+$ | ↓ in blood, liver and spleen<br>Static in lymph nodes |
| CCR6 | CD4$^+$ | No change |
| | CD8$^+$ | No change |
| CCR9 | CD4$^+$ | No change |
| | CD8$^+$ | ↑ in blood |
| B7integrin | CD4$^+$ | No change |
| | CD8$^+$ | ↑ in blood |
| CCR7$^-$/B7$^-$ (= surrogate for CXCR3 expressing cells) | CD4$^+$ | No change |
| | CD8$^+$ | ↓ in blood and liver<br>Static in lymph nodes and spleen |

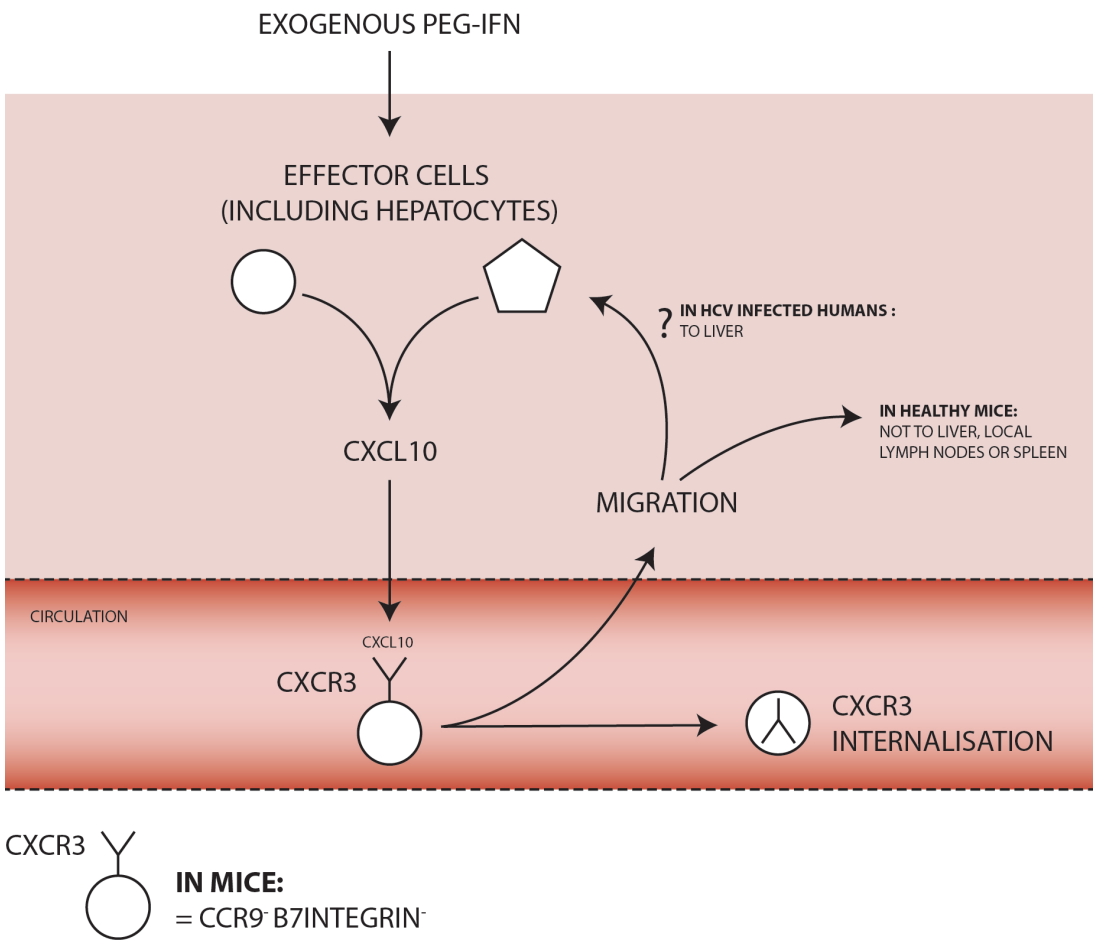
## 5.9 SUMMARY DISCUSSION

Key hypothesis

*Exogenously administered IFN-α drives hepatic recruitment of circulating CXCR3⁺ lymphocytes by induction of CXCR3-binding chemokines*

A summary of the key findings related to this hypothesis is presented in Figure 5-39.

Figure 5-39; Summary of findings related to interaction between exogenous interferon, effector cells and CXCR3 expressing lymphocytes




Exogenous PEG-IFN (pegylated interferon-alpha) stimulates effector cells, including hepatocytes to produce CXCL10. CXCL10 binds to the CXCR3 receptor on circulating lymphocytes to cause; i) CXCR3 receptor internalisation, and ii) migration of these lymphocytes from circulation. In mice, these cells preferentially migrate from circulation but not to liver, regional lymph nodes or spleen ($CCR9^-/B7^-$ integrin cells can be used as surrogates for this population).

In keeping with the key hypothesis of this chapter, 24 hours of PEG-IFN is first shown in-vivo to induce a significant increase in the plasma concentrations of the CXCR3-binding chemokines, CXCL10 and CXCL11, together with a substantial fall in circulating total lymphocyte number. Next, using IFN-α to stimulate hepatoma (Huh7.5) cells in-vitro, qPCR and ELISA demonstrate the liver is one source of CXCR3-binding chemokines.

Evaluating the in-vivo human data, there is a suggestion that the increase in plasma of both CXCL10 and CXCL11 negatively correlates with decreases in circulating lymphocyte number, but these analyses do not reach statistical significance, likely due to the small sample size evaluated. Concentrations of both CXCL11 and CXCL10 are next shown to correlate with CXCR3 expression on circulating $CD4^+$ and $CD8^+$ T cells at baseline. Furthermore, an inverse correlation between day 1 changes in CXCL10 and CXCR3 expression by $CD8^+$ T cells is demonstrated.

The observation that CXCR3 expression, together with plasma CXCL10 and CXCL11 concentrations return to baseline by week 4 in every patient studied suggests the profound induction of CXCR3-binding chemokines by PEG-IFN is only transient. Indeed, a subset of 3 patients for whom CXCL10 was evaluated longitudinally, 24 hours after the $2^{nd}$, $3^{rd}$ and $4^{th}$ doses of PEG-IFN demonstrated that, for CXCL10 at least, this effect is isolated to the first dose. The reason for this is unclear but may relate to PEG-IFN desensitisation through; i) ubiquitination and endocytosis of the IFN-α receptor chain 1 (IFNAR1) as a consequence of IFN-α mediated induction of the catalytic activity of protein kinase D2 (PKD2) (Zheng et al. 2011), and ii) negative regulation of the JAK-STAT pathway through suppressors of cytokine signaling (SOCS) (reviewed in Wormald 2003).

The clear in-vivo relationships that are demonstrated between CXCR3-binding chemokines and CXCR3 expression by $CD4^+$ and $CD8^+$ T cells, both at baseline, and during treatment are novel findings. Although plasma CXCL10 has generally been demonstrated to decrease during IFN-α therapy, the transient increase in plasma CXCL10 observed in the current study is not a completely novel finding - a recent paper by Albert and Colleagues found a similar increase in plasma CXCL10 levels, 6 hours after PEG-IFN administration in 5 patients (Casrouge et al. 2011). No previous data relating to changes in plasma CXCL11 early during therapy has been published.

Plasma levels of CXCL9, the only other CXCR3-binding chemokine, have not been evaluated in the current study. Nevertheless, based on my in-vitro qPCR data demonstrating a 5-fold increase in CXCL9 mRNA, it is highly likely that IFN-α similarly induces this chemokine in vivo.

Rather than inducing circulating $CXCR3^+$ lymphocyte redistribution, IFN-α may simply cause down-regulation of CXCR3 expression, or alternatively induce death of these lymphocytes. These possibilities were evaluated in Section 5.7 (page 236): IFN-α does cause down-regulation of CXCR3 expression by $CD4^+$ and $CD8^+$ T cells in-vitro, but not targeted death of this subpopulation. There was a non-significant trend suggesting in-vitro blocking of CXCL10 partially abrogated CXCR3 down-regulation –suggesting ligand binding driven by IFN-α may be an underlying mechanism behind this change.

Next using a murine model, the in-vivo effect of exogenous IFN-α on lymphocyte homing was further interrogated. In addition to CXCR3, IFN-α driven changes in expression of the chemokine receptors, CCR6, CCR9 and B7integrin by T cells was examined.

I found that mice treated with IFN-α had higher numbers of lymphocytes sequestered in their pre-auricular lymph nodes than control mice but observed no other changes in organ or circulating lymphocyte numbers. This contrasts with work by Kamphius and colleagues that demonstrated a fall in circulating lymphocytes 1 day after administering IFN to mice (Kamphuis et al. 2006).

Within the control group, PBMC counts for individual mice varied between $1.4 \times 10^6$ – $3.0 \times 10^6$ cells/ml ($1.9 \times 10^6 \pm 0.5 \times 10^6$ cells/ml (mean ± SD). This 'normal' variation is due, at least in part, to the technical challenge of isolating and quantifying lymphocytes from murine organs and blood. Unfortunately, this normal variation makes absolute quantification insensitive for the detection of subtle changes in lymphocyte subpopulations. Therefore, lymphocyte subpopulations were instead measured relative to their parent lymphocyte population to help minimize inaccuracies introduced by quantification.

As summarised in Table 5-3, compared with control mice, the proportion of $CD8^+$ T cells expressing CXCR3 was lower in the blood, liver and spleens (but not pre-auricular lymph nodes) of mice treated with IFN-α. Similar differences were not observed for $CD4^+$ T cells. Taken in isolation, the lower CXCR3 expression on $CD8^+$ T cells could potentially be explained by 2 mechanisms:

i. IFN-α drives down-regulation of surface CXCR3 expression

and/or

ii. IFN-α drives migration of $CXCR3^+$ cells from blood, liver and spleen

If CXCR3 down-regulation was the sole mechanism for the observed change, how can the increase in circulating $CCR9^+$ $CD8^+$ and $B7^+$ $CD8^+$ T cells be explained? In the context of

there being no measurable increase in total PBMC number, one possibility would be that IFN-α induces CCR9 and B7 integrin expression on $CD8^+$ T cells – however, ex-vivo stimulation of PBMCs with IFN-α did not induce expression of these chemokine receptors (Section 5.7, page 236). Instead, it is more likely, that a combination of the 2 mechanisms outlined above explains the observed change in CXCR3 expression. Migration of circulating $CXCR3^+$ lymphocytes from blood, would lead to a complementary increase in the proportion of circulating $CXCR3^-$ $CD8^+$ T cells which are high in CCR9 and B7integrin expression. This would explain the proportional increase in $CCR9^+$ and $B7integrin^+$ populations observed.

For murine $CD8^+$ T cells, FACS analysis demonstrated that 2 distinct populations are defined by cells that either; i) co-express CCR9/B7, or ii) express CXCR3. At baseline, $CCR9^-/B7^-$ $CD8^+$ T cells almost universally express CXCR3 and these can therefore be used as a surrogate population for $CXCR3^+$ $CD8^+$ T cells. This approach allowed the confounding factor of CXCR3 receptor down-regulation to be overcome when evaluating $CXCR3^+$ cell migration in response to IFN-α. As expected IFN-α caused circulating $CCR9^-/B7^-$ $CD8^+$ T cells to decrease but surprisingly, and in conflict with the primary hypothesis of this chapter, this cell population was also reduced in the liver of IFN-α treated mice.

Several fundamental differences were apparent comparing murine to in-vivo human findings that impact significantly on the translation of this model; in mice, unlike humans, i) CCR9 is expressed by substantially greater numbers of $CD4^+$ and $CD8^+$ T cells, and ii) a clear delineation can be made between $CCR9^+/B7^+$ and $CXCR3^+$ $CD8^+$ T cell populations.

So then, what can be concluded from this murine model? Firstly, the finding of an absolute increase in lymphocyte number in pre-auricular lymph nodes, in the absence of a local enrichment of $CXCR3^+$ T cells, suggests exogenous IFN-α drives lymph node sequestration

through a mechanism that is not CXCR3 dependent. Alternative mechanisms that might explain this phenomenon include IFN-α mediated up-regulation of CD69, with consequent impairment of lymphocyte egress from secondary lymphoid organs through S1P1 complexing (Shiow et al. 2006). Secondly, although IFN-α leads to CXCR3 down-regulation, using $CCR9^{-}/B7^{-}$ $CD8^{+}$ T cells as surrogates for $CXCR3^{+}$ lymphocytes, this study demonstrates these cells preferentially migrate from peripheral circulation, but not to the liver. Based on the available data, pre-auricular lymph nodes and spleen can also be excluded as sites of preferential $CXCR3^{+}$ T cell migration. Other possible sites of sequestration not evaluated in the current study include gut, loco-regional lymph nodes and lung.

One fundamental difference between this murine model and patients with chronic HCV infection that may account for the failure to observe hepatic enrichment of $CXCR3^{+}$ T cells is that of hepatic inflammation – present in HCV infected patients but not in healthy mice. It is possible that hepatic inflammation influences cellular migration through endogenous, local induction of chemokine release that is then further enhanced by IFN-α therapy.

During the course of this investigation, the substantial difference between my simple murine model and HCV infected patients on therapy became increasingly apparent. Although it would be interesting to know where $CXCR3^{+}$ cells are migrating to in healthy mice, the ability to subsequently translate such information into useful data for our HCV infected patients is questionable – for this reason, the model was not investigated further.

## 5.10 CONCLUSION

We can conclude that in humans, IFN-α transiently induces CXCR3-binding chemokines in association with a decrease in circulating lymphocyte number. Although CXCR3 receptor down-regulation may partly account for this change, this population also preferentially migrates from circulation – it remains unclear to where these cells are migrating to – in healthy mice at least, we can exclude liver, pre-auricular lymph nodes and spleen.

# Chapter 6 Concluding remarks

This thesis explores, for the first time in man, a combination of heterologous adenovirus and MVA vector based vaccines targeting hepatitis C virus infection. The first investigative chapter details the clinical application of these vaccines together with an interrogation of the immune responses they generate in HCV infected patients. The second investigative chapter broadly explores baseline and treatment induced differences in lymphocyte subpopulations that might explain the reason for attenuated responses to vaccination observed in those with chronic HCV infection. And finally, the third investigative chapter focuses on the hypothesis that HCV treatment induces chemokine mediated hepatic recruitment of lymphocytes – a phenomenon that might not only have explained lymphopenia during therapy, but also provided an indirect means of improving delivery of vaccine induced T cells to HCV infected hepatocytes.

In the first investigative chapter (Chapter 3), I show that T cell responses can be safely induced by vaccination in HCV infected patients. However, compared with healthy volunteers, responses to vaccination in patients with chronic HCV infection are reduced in their frequency, magnitude and breadth. Detailed interrogation of these responses in HCV infected patients indicates that the epitopes targeted by vaccine induced T cells do not correspond to those of autologous circulating virus. In addition, at epitopes where autologous virus does match vaccine immunogen, in no cases did vaccination successfully induce T cell responses.

Why should vaccine induced T cells not target circulating virus? Exploring sequence variability at a population level for immunogenic epitopes indicates that for certain

epitopes, vaccine immunogen actually represents a rare HCV variant. In these cases, T cells successfully induced by vaccine immunogen will not target commonly circulating virus. However, this is explanation cannot be universally applied – some epitopes in the vaccine immunogen are highly conserved – and although T cells targeting a conserved epitope were only induced in 1 patient, the autologous virus in this patient represented a rare escape variant. Whether this sequence variation occurred by chance or as a consequence of natural immune pressure (before vaccination) remains the focus of ongoing work.

The differences observed between vaccine immunogen and common HCV sequences at immunogenic epitopes highlights the importance of rational immunogen design. A vaccine insert that contains rare epitope variants that induce T cells with limited ability to cross-recognise common sequences is clearly not ideal. As a next step towards an effective therapeutic vaccine, careful selection of vaccine immunogen should be a priority.

There are several approaches that could be taken to design a more effective vaccine insert. Like many others, our HCV003 vaccine approach has focused on inducing a breadth of T cell responses (using an insert selected from a single genotype 1b HCV sequence). Theoretically, delivery of an appropriately sized insert will induce a variety of T cells that recognise a range (or breadth) of different epitopes - and ultimately only a selection of these will target circulating virus. Clearly this approach, at least in the setting of chronic HCV infection may not be a realistic goal – delivery of our insert was at best able to induce T cell responses to 3 different peptide pools in a single patient, and our typical vaccine responder developed novel T cells targeting just 1 epitope. It should be noted that this was not the case for healthy volunteers who typically developed T cell responses across a broad range of T cell epitopes.

Recently, it has been suggested that rather than breadth of vaccine response, rational design should focus on the depth of vaccine induced responses, where depth is defined by the number of different variants of a single epitope recognised (Hanke & McMichael 2011). Here, the vaccine insert might consist of a mosaic of variants to a single, or limited number of immunogenic epitopes. Such an approach would theoretically limit the capacity of HCV to escape from T cell recognition at these individual epitopes. However, the same problem observed with limited breadth might well apply to the depth of responses in those vaccinated during chronic HCV infection – T cells only recognising 1 variant or a few variants might be induced despite a mosaic insert - partly because certain epitope variants may be immunodominant, and partly as a consequence of the negative immunomodulatory effect of chronic HCV infection itself.

Even if a vaccine insert capable of inducing T cells that recognise autologous virus can be successfully designed, a second and just as important hurdle for an effective therapeutic vaccine is the immunomodulatory effect of chronic HCV infection itself, together with concurrently administered PEG-IFN/ribavirin induced changes. Based on the HCV003 data (together with that of HCV002), there is a suggestion that T cells recognising immunodominant viral epitopes may be present before vaccination in low frequency, but as a result of chronic antigen exposure they are exhausted and non-functional. If this is the case, rather than inducing novel responses with vaccination, we may only be boosting pre-existing, but ineffective responses. Phenotypic data indicate that this may well be the case – vaccine induced T cells are typically high in the exhaustion marker (PD-1) and proliferate poorly. Nevertheless, T cell exhaustion might be combated using a variety of strategies such as PD-1 blockade (Finnefrock et al. 2009).

So then, is there another approach that could be used? Rather than choosing previously identified immunodominant epitopes to which T cells are likely to have been already primed and exhausted, if instead our vaccine insert contained only subdominant epitopes that were highly conserved, it is possible that the problems of breadth, depth and exhaustion might be overcome. Vaccination would theoretically prime naïve, functional T cells to epitopes matching autologous virus. A mouse model of HIV DNA vaccination shows manipulation of the T cell dominance hierarchy is possible. In this study removal of dominant epitopes in a vaccine immunogen facilitated the induction of T cell immunity to subdominant epitopes (Im et al. 2011). Identifying HCV regions using computer algorithms that are conserved between different virus genotypes and contain subdominant T cell epitopes is an approach currently being explored (Dr Annette Bohmer, DPhil student of Dr E. Barnes).

Even with the rational design of a new insert, the effect of chronic HCV infection on vaccine immunogenicity, together with the value of concurrent traditional antiviral therapy needs to be considered. There is evidence from others, at least in a mouse model, that reducing viral load in the setting of chronic infection improves vaccine immunogenicity and this was the reason for delivery of vaccine in 1 arm of the HCV003 trial during PEG-IFN/ribavirin therapy (Wherry & Ahmed 2004).

Although the HCV003 study was not powered to detect a difference, comparing patients across groups suggests that PEG-IFN/ribavirin therapy may have actually impaired vaccine responses, rather than enhancing them– only 2/7 recipients in Arm B (on therapy) developed novel T cell responses compared with 3/4 of those who received vaccination alone. In addition to this observation, recently published work has indicated that PEG-IFN causes lymphopenia in a significant number of patients and somewhat surprisingly, these

patients have better treatment outcomes. For these reasons, exploring the differences in lymphocyte subpopulations in chronic HCV infection together with the effects of PEG-IFN/ribavirin on these cells became the focus of the second investigative chapter of this thesis.

In the second investigative chapter (THIRST trial), I established a separate cohort of patients who were followed closely during the first four weeks of PEG-IFN/ribavirin therapy and compared these with healthy controls. A series of flow cytometry panels were developed to broadly assess differences in circulating lymphocyte subpopulations together with changes in their homing capacity, activation, differentiation, and cytokine profiles.

At baseline in the THIRST trial, and in keeping with previously published data, I found HCV infected patients had higher proportions of regulatory T cells in circulation - although this is not a new finding, it nevertheless provides a potential explanation for the reduced immunogenicity of vaccination in patients compared with healthy individuals. Similarly, the relative increases in circulating $NK^{bright}$ cells is not a new finding but suggests clear differences in innate immune function during chronic infection that might further impact on vaccine responses.

It is difficult to interpret the significance of the complex changes in cytokine production by $CD4^+$ and $CD8^+$ T cells that I found in patients with chronic HCV infection compared with healthy controls. I found that PEG-IFN/ribavirin therapy increases IFN-γ production globally by T cells upon PMA/ionomycin stimulation. Although early data from the 1980s supports this finding, the mechanism behind this is unclear (see page 195). Furthermore, my findings relating to increased IL-2 and TNF-α production at baseline are unique. It is

surprising that HCV should cause such global change in cytokine production, and I plan to validate these findings in a larger cohort before exploring this further.

As expected, in the THIRST trial, PEG-IFN/ribavirin therapy caused lymphopenia in most patients. In addition, for the first time in-vivo, I demonstrate that therapy causes transient activation of T cells (up-regulation of CD69, HLA-DR and CD38 on day 1) and alters their cytokine profiles, with significant increases in IL-17 production, contrasting with decreasing IFN-γ production over 28 days of therapy. Furthermore, and perhaps most interestingly decreases in the proportion of circulating lymphocytes that express the liver homing chemokine receptors CXCR3 and CCR6 are observed transiently 1 day after starting therapy. Interrogation of receptor co-expression indicated that this was a CXCR3 mediated effect because there was no change in circulating $CCR6^{+}/CXCR3^{-}$ cells at this early time-point.

How do these findings impact on the application of a therapeutic vaccine and can they be used in any way to improve vaccine responses? One could argue that in some ways, they are redundant – vaccine responses in the HCV003 trial (together with data recently presented relating to another viral vectored HCV vaccine (Wedemeyer et al. 2012)) indicate that PEG-IFN/ribavirin therapy impairs therapeutic vaccine responses. Consequently, future therapeutic HCV vaccine trials may universally explore vaccine responses without concomitant PEG-IFN/ribavirin therapy. It is possible however, that IFN-γ ELISpot data based on T cells in circulation does not accurately reflect the true response to vaccination because therapy or HCV infection itself might cause hepatic sequestration of vaccine induced T cells. This possibility is highlighted by the finding that during therapy lymphopenia occurs early, as does a decrease in lymphocytes expressing liver homing chemokine receptors.

In the THIRST trial, the substantial number of comparisons made between groups does raise the possibility of type 1 statistical errors - where differences that are attributed statistical significance are actually just rare events that have occurred by chance. The application of a Bonferroni correction, or other similar measure to reduce the chance of a type 1 error generally requires that the hypotheses being tested are independent from one another – this was not the case for comparisons made in the THIRST trial. Furthermore, such corrections have their own limitations: setting a more stringent p value (using a Bonferroni correction) might introduce a type 2 statistical error, where biological differences between groups are missed. For this reason, the data was analysed without these corrections.

The third investigative chapter of this thesis explores the intriguing possibility that therapy induces chemokine mediated lymphocyte sequestration in detail. CXCL10, one chemokine that binds CXCR3 has been previously shown to be elevated in the plasma of patients with chronic HCV infection. Historically, CXCL10 was thought to play no role in PEG-IFN/ribavirin mediated viral clearance because levels have generally been shown to fall during therapy. Nevertheless, no previous data examining CXCL10 levels early after commencing therapy had been published (a paper published in 2011 has now reported this (Casrouge et al. 2011)).

I first evaluated changes in plasma CXCL10 in patients enrolled in the THIRST study together with samples from another 3 patients who had longitudinal sampling 1 day after their 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ PEG-IFN doses. A profound increase in CXCL10 occurred 24 hours after the first dose of PEG-IFN. Furthermore, this was found to inversely correlate with decreases in circulating CXCR3$^{+}$ CD8$^{+}$ T cells.

Due to the significant morbidity and risk attached to longitudinal liver biopsies, it is not possible to evaluate hepatic sequestration of lymphocytes in humans during HCV therapy. Additionally, through a series of in-vitro experiments I found that, although hepatocytes are one source of CXCL10, IFN-α also causes down-regulation of surface CXCR3 expression by lymphocytes. This suggests the possibility that the original changes observed in-vivo during HCV therapy might not be related to lymphocyte redistribution but rather changes in receptor expression.

For these reasons, I next evaluated organ sequestration of lymphocytes driven by IFN-α in a mouse model. This did not prove to be as simple as I had hoped. While I found that IFN-α caused a decrease in CXCR3 expressing lymphocytes in circulation, no hepatic enrichment was demonstrated. Furthermore, there was no evidence that total lymphocyte number increased in the livers of IFN-α treated mice. Instead, I found an increase in pre-auricular lymph node cell number that was not $CXCR3^+$ lymphocyte predominant. The possibility that CXCR3 down-regulation was masking enrichment of this lymphocyte population was next considered - through detailed evaluation of gut homing chemokine receptors CCR9 and B7integrin, I discovered that in mice, $CCR9^-$/$B7integrin^-$ cells could be used as surrogates for $CXCR3^+$ lymphocytes.

Evaluating the $CCR9^-$/$B7integrin^-$ population, I first demonstrate that IFN-α does not cause hepatic enrichment of these cells (and therefore $CXCR3^+$ cells) in a healthy mouse model. It is however clear that this population does migrate from circulation – but not to any organ (liver/spleen) or lymph node (pre-auricular) assessed. There are a number of other experiments that could be employed to further investigate where lymphocytes are migrating to in IFN-α treated mice. For example, mice with luciferase reporter labeled

lymphocytes could be employed to track lymphocytes in-vivo. However, the increasing complexity of the experiments required to investigate this question further, also highlighted the substantial differences between the simple murine model and HCV infected patients. Perhaps most importantly, the clear differences in chemokine receptor co-expression between murine and human lymphocytes that I discovered, together with the obvious difference of hepatic inflammation – present in HCV infection, but not healthy mice. For these reasons, although the original question remains intriguing, I did not investigate it further.

Finally, there are currently a plethora of small molecule, rationally designed HCV therapies in clinical development, many of which promise IFN-α free treatment with high treatment success rates in the next 5-10 years. So then, why should we bother with a therapeutic vaccine at all? In its current form, the vaccine shows little promise as an effective treatment for HCV infection. Even with the rationale design of a new insert, successful therapeutic vaccination is still likely to remain a substantial challenge. Nevertheless, for 'difficult to treat' patients, especially those with advanced fibrosis, therapeutic vaccination remains an attractive goal. In addition, perhaps the best support for the value of this research comes from the fact that a phase 2 trial, evaluating AdCh3-NSmut/MVA-NSmut vaccination for prophylaxis against HCV infection has recently commenced (Clinical trial id: NCT01436357). Our detailed evaluation of vaccine responses in patients with chronic HCV infection has not only helped inform this phase 2 trial, but highlighted potential obstacles, such as the intrinsic variability of viral sequence that are also likely to challenge the effectiveness of the current vaccines.

Although I have provided further evidence that therapeutic vaccination for hepatitis C is a realistic goal, this work has also highlighted a number of obstacles that will need to be

tackled for such an approach to be carried forward. It seems that the more we dig, the more complex the treasure map becomes! Or perhaps… I wonder if it is finally becoming clearer to me – it's not just about the treasure at the bottom of the hole, but also the unexpected gems one finds along the way.

Thank you for taking the time to read this work.

Sincerely,

John Halliday, Oxford, Dec. 2012

# Chapter 7 Appendices

## Appendix 1 - Consent form for HCV003 trial

**CONSENT FORM**

**Title of Project:** A Phase I study to assess the safety and immunogenicity of AdCh3NSmut and MVA-NSmut in healthy volunteers and patients with hepatitis C virus infection

**Short title:** Study of a new MVA vaccine for hepatitis C virus

**Chief Investigator:** Dr. Eleanor Barnes

**Study Code:** HCV003 **OxREC A ref. number:** 10/H0604/45

**Subject ID:** HCV003-_____

***Please initial** (right) **and sign** (bottom)*

| | | |
|---|---|---|
| **1** | I confirm that I have read and understand the Patient Information Sheet Version_____ dated_____(to be completed by volunteer) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. | |
| **2** | I have spoken to:_____ | |
| **3** | I understand that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. | |
| **4** | I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals identified by Okairos (sponsor), the clinical trial monitors and regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. | |
| **5** | I agree to my GP being contacted regarding my participation in the study. | |
| **6** | I agree that some of my leftover blood samples will be stored for up to 15 years and may be used for further ethically approved studies of the body's immune response to hepatitis C virus (HCV) vaccination, or stored in a research tissue bank to be used for future ethically approved research. *(Your ability to participate in this study will not be affected by your decision to allow or not allow storage and future use of your leftover blood samples).* | |
| **7** | I agree to medical screening, including testing for HIV and Hepatitis B virus and Hepatitis C virus | |
| **8** | If I am in Arm B, I understand the need to use an effective method of barrier contraception during the trial and for 6 months after my last dose of ribavirin. | |
| **9** | If I am in Arms A or C, I agree to use effective contraception and not get pregnant for the duration of the trial. If I am a man I will use barrier contraception until 3 months after the last vaccine. | |
| **10** | I agree not to donate blood for the duration of the trial (or for life if I have hepatitis C virus infection) | |
| **11** | I agree to allow photographs to be taken for clinical comparison, and I understand I will not be identifiable in these photographs other than by the unique study number. | |
| **12** | If I am in Arm A, I agree to be registered with a confidential database to prevent entry into other clinical trials at the same time as I am participating in this study | |
| **13** | I agree to take part in this study. | |

**Signed:** ..... **Date:** .....

**Name:** **(in block letters)**.....

**Signature of Investigator:** ..... **Date:** .....

**Name of Investigator:** **(in block letters)** .....

## Appendix 2 – Patient information sheet for THIRST study

Oxford Radcliffe Hospitals NHS
NHS Trust

Hepatobillary Unit
John Radcliffe Hospital
Headley Way
Headington
OX3 9DU

Tel: 01865 220944
Fax: 01865 220955

**Patient Information Sheet,**
**Version 4, October 2008,**
**Study No: 04OXA.010**

**A Study of the immune responses during and after liver liver inflamation**

***What is the purpose of the study*?**

Liver inflammation can be caused by a variety of factors, including viral infection. We would like to know why some patients are able to control the inflammation completely and others develop liver damage, and also why some patients respond to treatment and others do not. Understanding the immune responses in these situations would help to design vaccines in the future or potentially other forms of therapy.

***Why have I been chosen*?**

You have bee chosen because you are attending/have attended a clinic specialising in liver diseases.

***Do I have to take part***?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time or a decision not to take part, will not affect the standard of care you receive.

***What will happen to me if I take part?***

The study would involve giving extra blood samples at the time of your routine clinic samples or at another organised visit. Initally this would only be an extra 30-45mls of blood(3-5 tubes). There should be no side effects from this but occasionally brusing occurs. If individual patients had a particularly strong immune response, or if patients are going to be treated with specific therapies, we might ask to take further samples later. this is because we are interested in how the immune response changes with treatment.

Your blood samples would be stored anonymously for the duration of the project, at either the Peter Medwar building Oxford, or at the site of our international collaborators.

## Appendix 3 – Patient consent form for THIRST study

Oxford Radcliffe Hospitals NHS
NHS Trust

Hepatobillary Unit
John Radcliffe Hospital
Headley Way
Headington
OX3 9DU

Tel: 01865 220944
Fax: 01865 220955

**Patient Information Sheet,**
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***Do I have to take part***?

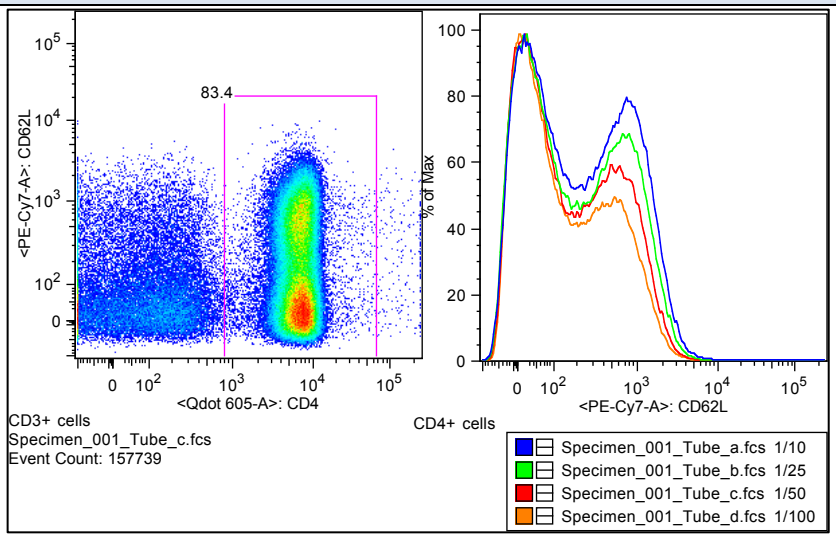
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time or a decision not to take part, will not affect the standard of care you receive.

***What will happen to me if I take part?***

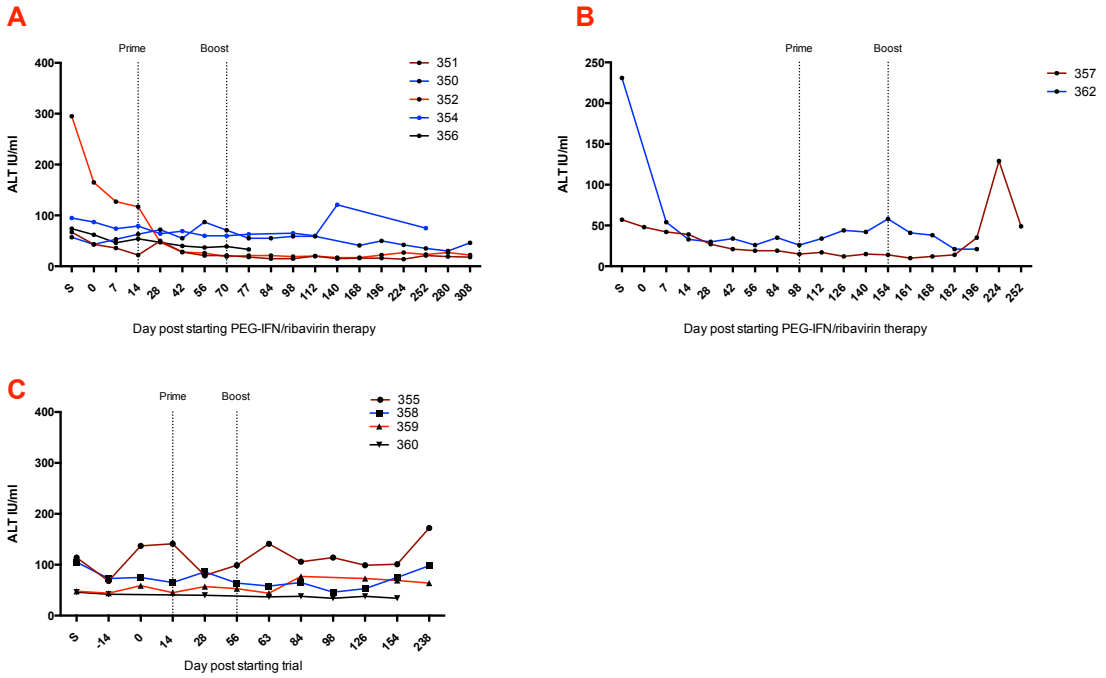
The study would involve giving extra blood samples at the time of your routine clinic samples or at another organised visit. Initally this would only be an extra 30-45mls of blood(3-5 tubes). There should be no side effects from this but occasionally brusing occurs. If individual patients had a particularly strong immune response, or if patients are going to be treated with specific therapies, we might ask to take further samples later. this is because we are interested in how the immune response changes with treatment.

Your blood samples would be stored anonymously for the duration of the project, at either the Peter Medwar building Oxford, or at the site of our international collaborators.

## Appendix 4 Example FACS antibody titration

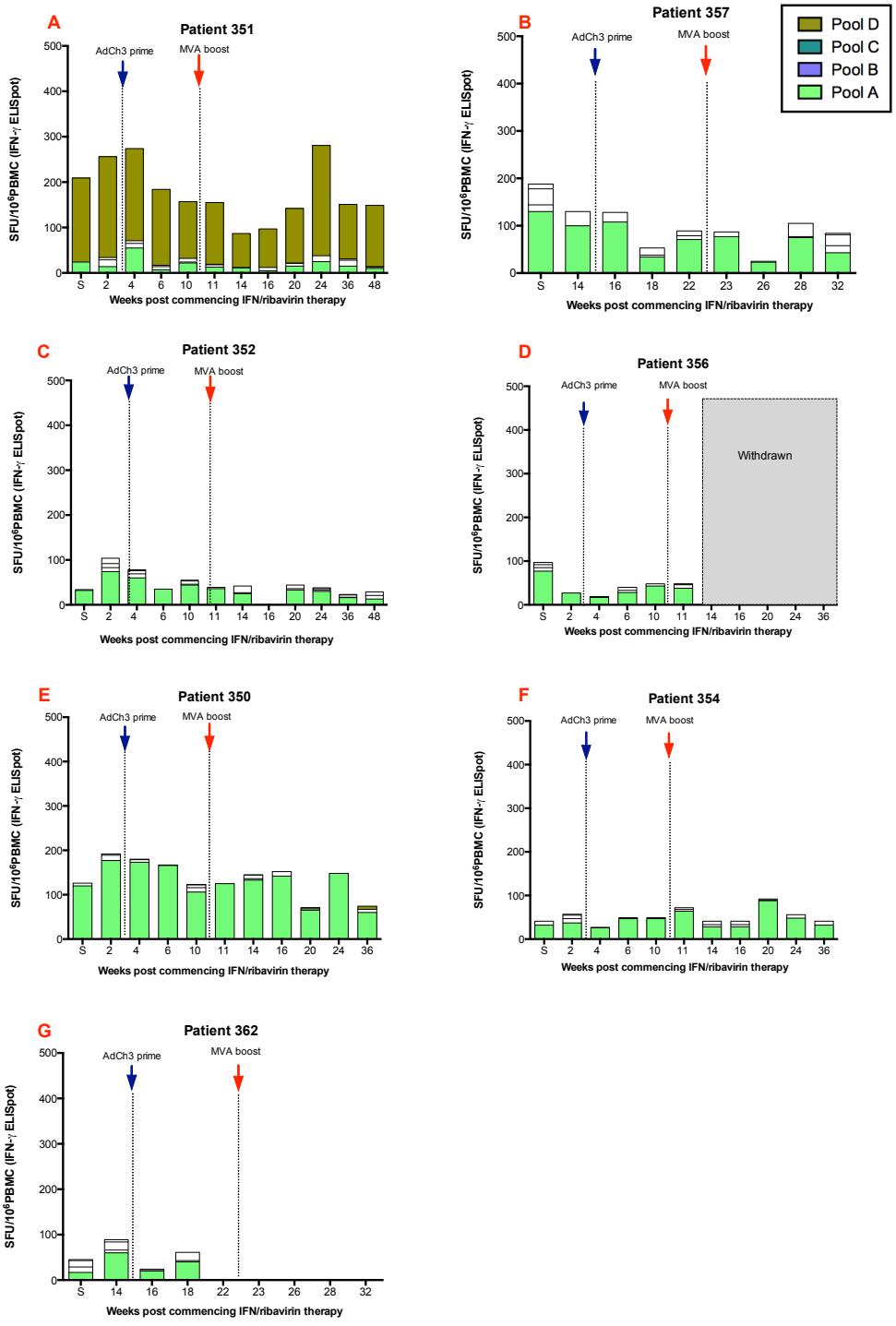


Appendix 5 Change in alanine aminotransferase (ALT) over time in HCV infected trial participants



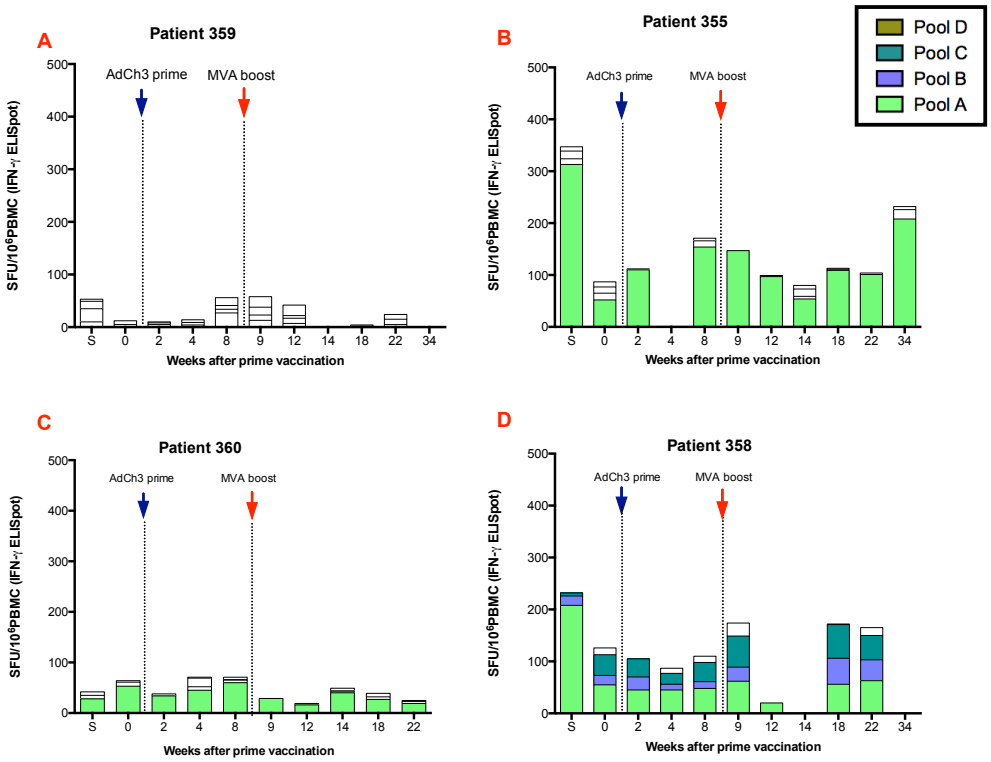

Graph A: Patients enrolled in arm B1. Graph B: Patients enrolled in arm B2. Graph C: Patients enrolled in arm C. ALT; Alanine aminotransferase. Time of prime/boost vaccination is indicted by vertical dotted line. Each coloured line connects ALT levels in individual trial participants across time.

Appendix 6 IFN-γ ELISspot responses to HCV peptide pools not included in the vaccine insert over time in individual patients in Arm B



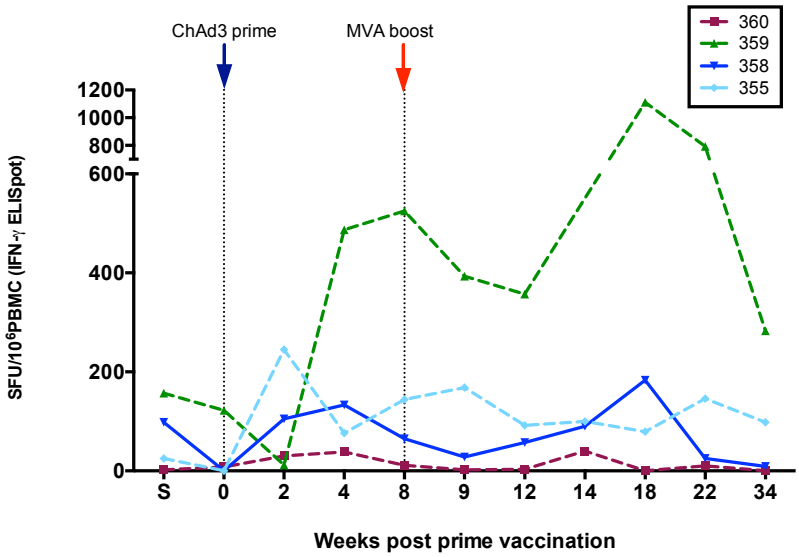

Responses are summed responses to pools containing NS proteins in the vaccine immunogen. Only positive pools (defined as >41 SFU/$10^6$ PBMCs) are shaded in colour. Those pools with responses that fell below the positive cut-off in individual patients are left unshaded.

Appendix 7 IFN-γ ELISspot responses to HCV peptide pools not included in the vaccine insert over time in individual patients in Arm C



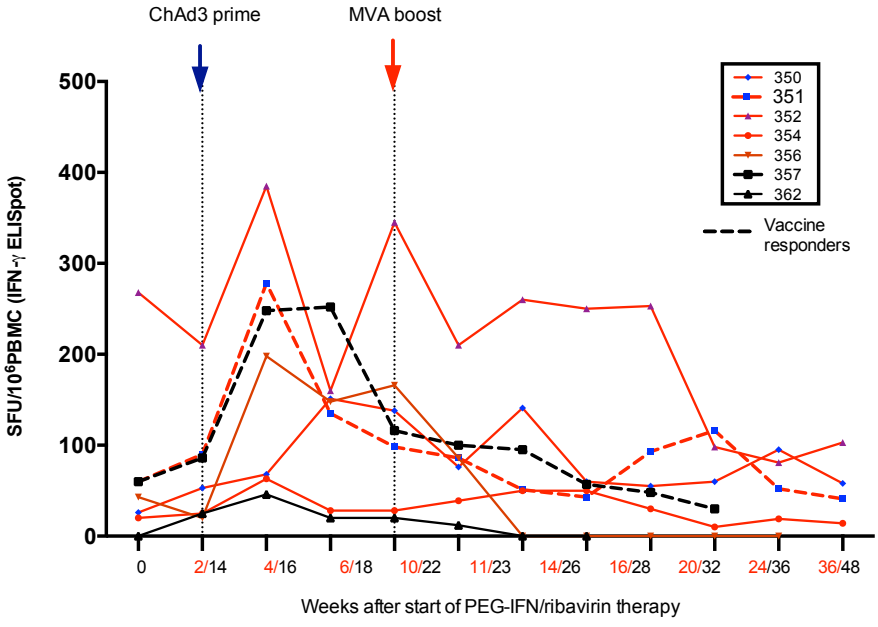

Responses are summed responses to pools containing NS proteins in the vaccine immunogen. Only positive pools (defined as >41 SFU/$10^6$ PBMCs) are shaded in colour. Those pools with responses that fell below the positive cut-off in individual patients are left unshaded.

## Appendix 8; IFN-γ ELISspot responses to Ad5 Hexon over time for patients in Arm C



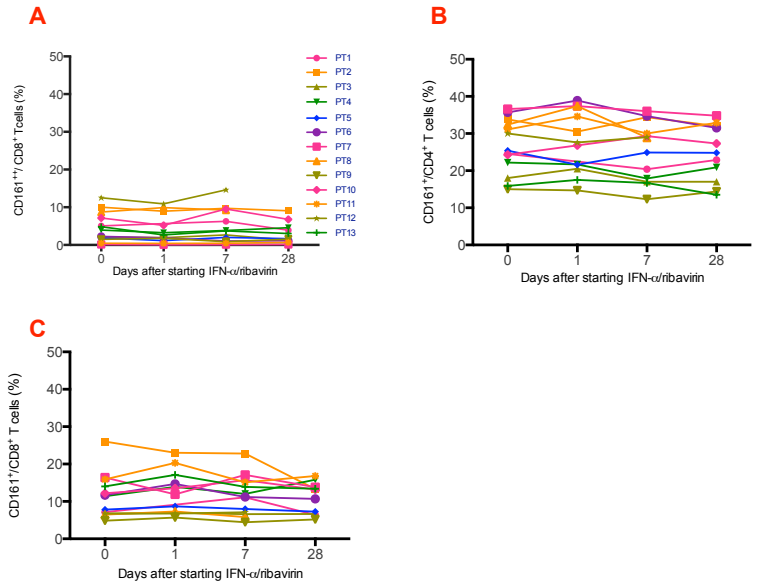

Prime vaccine = ChAd3-NSmut. Boost vaccine = MVA-NSmut. Kinetics of ex vivo IFN-γ ELISpot responses over time by individual. These patients (Arm C) received vaccination alone without concurrent anti-HCV therapy. Coloured lines connect individual patient responses over time. T cell responses represent responses to the Ad5 Hexon pool. S=screening. Dashed lines represent individuals who were classed as vaccine responders (based on novel T cell responses to vaccine immunogen. Ad5 = human adenovirus serotype 5

## Appendix 9; IFN-γ ELISspot responses to Ad5 Hexon over time for patients in Arm B

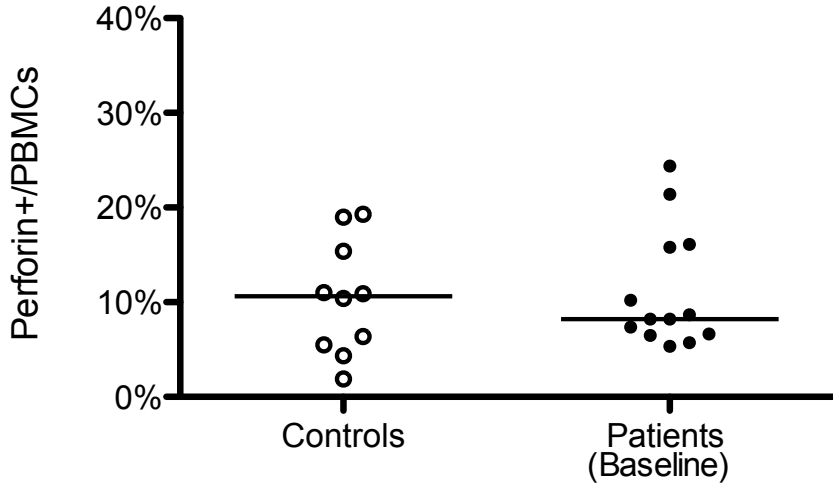



Kinetics of ex-vivo IFN-γ ELISpot responses over time for individual HCV003 patients. These patients (Arm B) received prime vaccination either after 2 weeks therapy (red lines) or 14 weeks (black lines) PEG-IFN/ribavirin therapy, followed 8 weeks later by boost vaccination. T cell responses are responses to the Ad5 hexon pool. Dotted vertical lines indicate timing of prime and boost vaccination. Dashed lines represent individuals who were classed as vaccine responders (based on novel T cell responses to vaccine immunogen.

Appendix 10; Longitudinal change in CD161⁺ T cells for individual patients enrolled in the THIRST trial

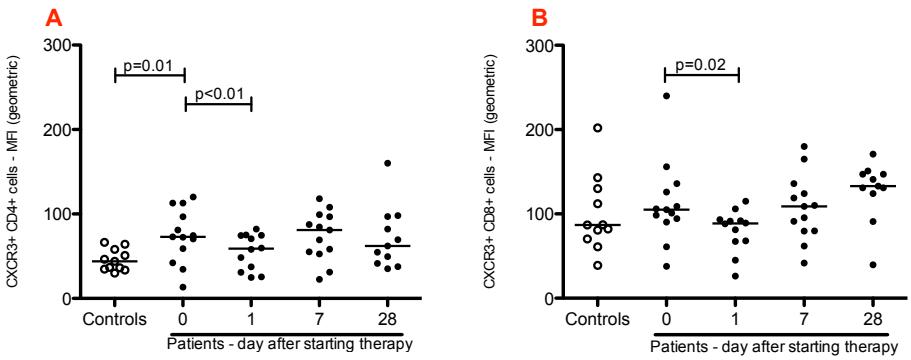


Appendix 11; Perforin+ CD3+ T cells as a proportion of PBMCs



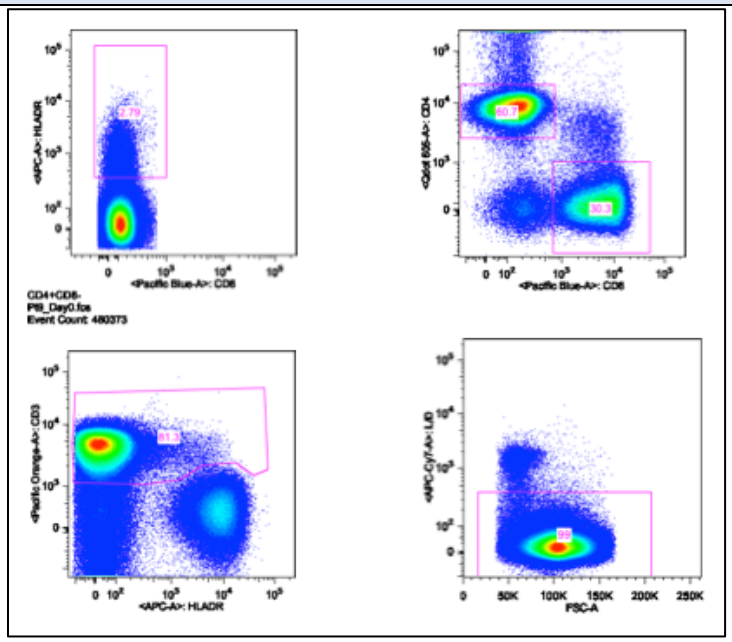

Healthy controls compared with HCV infected patients at baseline. Horizontal lines depict median values.

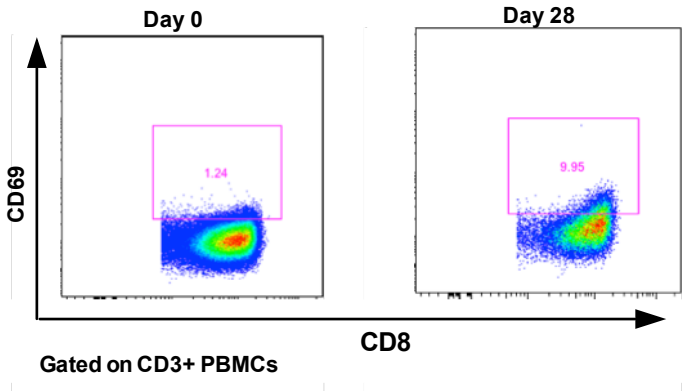
Appendix 12; Geometric median fluorescence intensity for CXCR3+ CD4+ and CD8+ T cells




Control values are normalised against the median of all day 0 patient samples (unpaired). Day 1, 7 and 28 values represent the absolute change from day 0 for each individual patient (paired). Horizontal lines represent median values.

Appendix 13; Example Gating strategy for HLA-DR

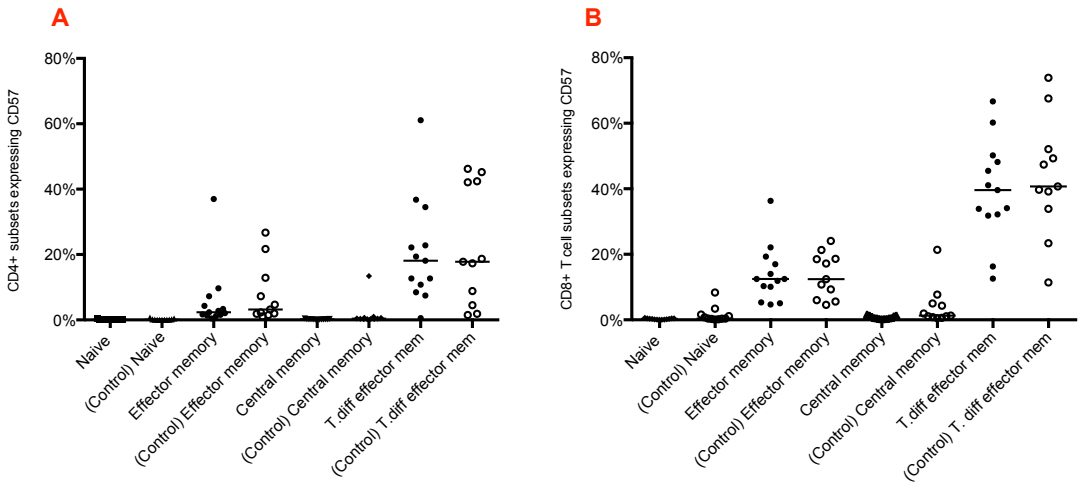



Appendix 14

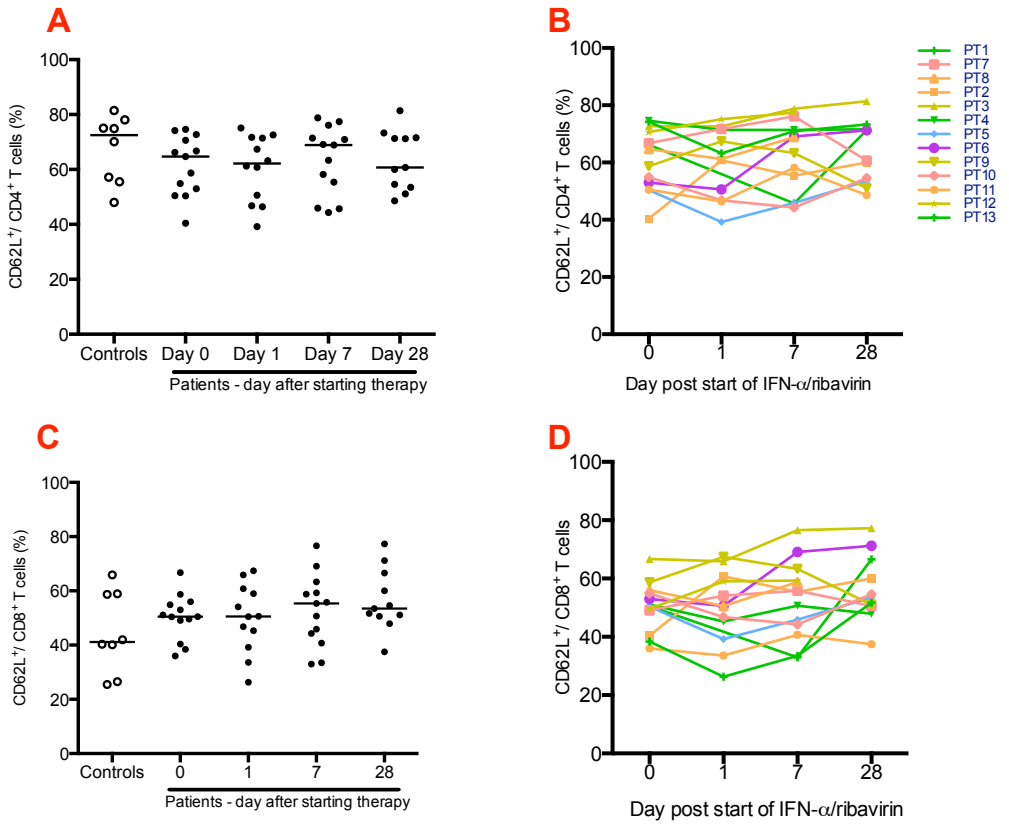
FACS plots demonstrating CD69 gating and longitudinal change between day 0 and day 28 for an individual patient

Appendix 15; CD4 and CD8 memory subsets expressing CD57 at baseline



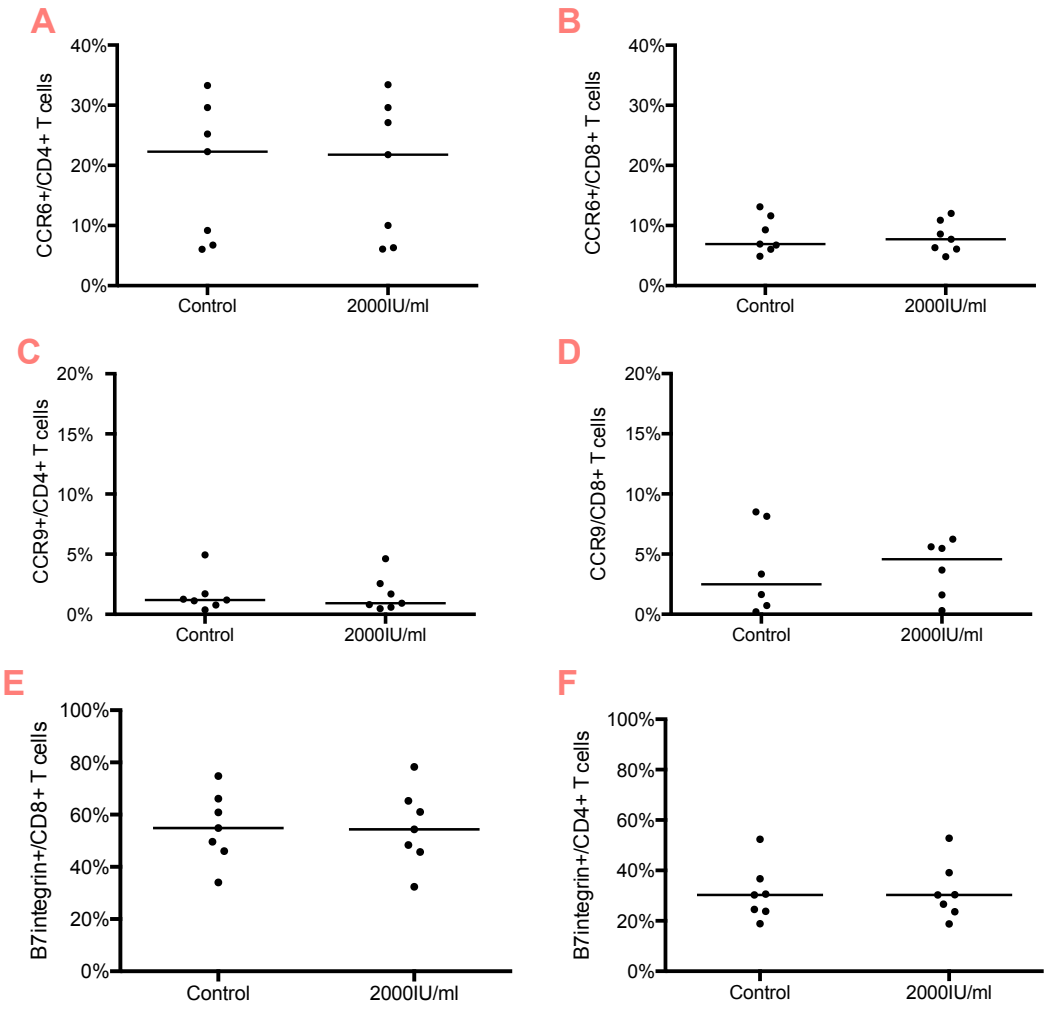

CHC patients compared with healthy controls at baseline. $CD4^+$ T cells (A) and $CD8^+$ T cells (B). Horizontal lines depict group medians.

Appendix 16; Longitudinal changes in CD62L expression during therapy in HCV infected patients compared with healthy controls

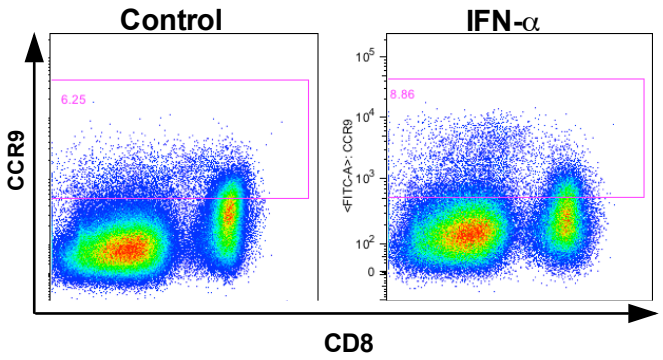



Top row: $CD4^+$ T cells. Bottom row: $CD8^+$ T cells. Right column – coloured lines connect individual patient samples across time. Left column – Absolute % of control values together with baseline and longitudinal values during therapy. Horizontal lines depict median values.

Appendix 17 Changes in CCR6, CCR9 and B7 integrin by IFN-α stimulated T cells




Cells were stimulated at concentrations of 0 IU/ml (control), and 2000 IU/ml for 24 hours. Plots represent samples from 7 individual healthy volunteers. The left column of graphs represents $CD4^+$ T cells and the right column represents $CD8^+$ T cells. (A&B) represent CCR6 (C&D) represent CCR9 and (E&F) represent B7integrin expression. Horizontal lines depict group medians.




Appendix 18; Example FACS plots of CCR9 expression on lymphocytes ($CD8^+$ and CD8 – T cells) isolated from the liver in 2 individual mice

Control mouse (left plot) and an IFN-α receiving mouse (right plot). Gated on single cells/MNCs/live cells/$CD3^+$

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