



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

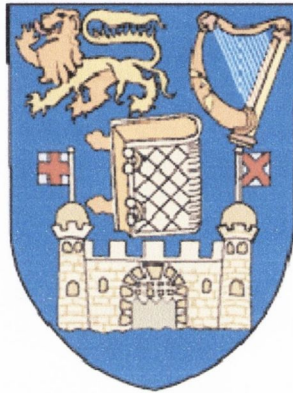
By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

HCV evasion of IFN- α signalling: implications for therapy



**This thesis is submitted to the University of Dublin for the
degree of Doctor of Philosophy**

Nollaig Bourke (BA mod)

School of Biochemistry and Immunology

Trinity College Dublin

January 2012

Supervisors: Prof. Cliona O'Farrelly and Dr. Nigel Stevenson



Thesis 9654

Declaration

I hereby certify that this thesis has not been previously submitted for examination to this or any other university. The work described herein was carried out by the author alone unless otherwise stated, with the exception of Fig. 3.12, Fig. 4.18 and Fig. 4.19, of which Dr. Nigel Stevenson contributed 50% of the work.

The author gives permission for the library to lend or copy this work upon request.



Nollaig Bourke

January 2012

Tables of Contents

Tables of Contents.....3

List of Tables.....11

Acknowledgements.....12

Publications and funding.....14

Abbreviations.....15

Abstract.....21

Chapter 1 Introduction.....24

 1.1 Hepatitis C Virus26

 1.1.1 Structural proteins27

 1.1.2 Non-structural proteins30

 1.1.3 HCV viral life cycle33

 1.1.4 HCV Genotypes and quasispecies35

 1.1.5 History of HCV infection in Ireland36

 1.2 Anti-viral immunity.....38

 1.3 The anti-viral innate immune system.....41

 1.3.1 Innate immune cells involved in anti-viral immunity.....41

 1.3.2 Pattern Recognition Receptors (PRRs).....42

 1.3.3 Viral recognition by the innate immune system.....44

 1.4 Interferons.....46

 1.4.1 Type I IFNs effector functions47

 1.4.2 Type I IFNs and the adaptive immune response47

 1.4.3 Type I IFN receptor.....48

 1.5 The JAK-STAT pathway50

 1.5.1 JAK proteins52

 1.5.2 STAT proteins.....53

 1.5.3 STAT proteins and IFN- α54

 1.5.4 Anti-viral effector proteins: PKR, OAS and MxA.....55

 1.6 Viral immune evasion56

 1.6.1 Viral inhibition of TLR signalling.....56

 1.6.2 Viral inhibition of RLR signalling58

 1.6.3 Viral targeting of IFN signal transduction60

 1.6.4 Viral targeting of type I IFN induced effector molecules61

 1.7 HCV therapy: IFN- α 62

 1.8 Rationale and overall objective.....63

 1.8.1 Rationale63

 1.8.2 Hypothesis.....63

 1.8.3 Specific aims64

Chapter 2 Materials & Methods66

 2.1 Materials.....66

 2.1.1 General Materials.....66

 2.2.2 Cell lines/constructs66

 2.2.3 Antibodies67

 2.2.4 Cell culture reagents.....67

 2.2 Patient information.....67

 2.2.1 Patient recruitment67

 2.2.2 Ethical approval68

 2.2.3 Treatment Regime68

2.2.4 Antibody and PCR testing.....	68
2.2.5 Sample Collection.....	69
2.3 Methods.....	69
2.3.1 PBMC isolation.....	69
2.3.2 Culture of Huh-7 cell line	70
2.3.3 Stimulations	70
2.3.4 Peptide synthesis and reconstitution	70
2.3.5 Huh-7 cell transfection.....	71
2.3.6 RNA extraction and quality control	72
2.3.7 cDNA synthesis and qRT-PCR.....	74
2.3.8 Primer design	75
2.3.9 GeNorm analysis.....	75
2.3.10 Quantitative real-time polymerase chain reaction (qRT-PCR) data analysis	76
2.3.11 Sample preparation for western blot	76
2.3.12 Western blotting	77
2.3.13 Flow cytometry	78
2.3.14 Plasmid transformation	79
2.3.15 Plasmid preparation and purification	80
2.3.16 Agarose gels	81
2.3.17 Cloning.....	81
2.3.18 Gel Clean up.....	82
2.3.19 Luciferase reporter gene assay	83
2.3.20 Short hairpin RNA (shRNA) and small interfering RNA (siRNA)	83
2.3.21 Bioinformatics.....	84
2.3.22 Statistics	85
Chapter 3 HCV degrades STAT3 via the proteasome: identification of inhibitory peptides against viral targets that restore IFN- α responsiveness	87
3.1 Introduction.....	87
3.1.1 HCV and STAT proteins	88
3.1.2 Viral targeting of STAT proteins for degradation via the proteasome	89
3.1.3 Ubiquitination and proteasome-mediated degradation	89
3.1.4 Virally encoded E3 ligases with VHL- and SOCS- boxes.....	91
3.1.5 Aims	93
3.2 Results.....	94
3.2.1 STAT1 and STAT3 protein expression is reduced by HCV	94
3.2.2 STAT1 and STAT3 mRNA is unaffected by HCV expression	95
3.2.3 IFN- α stimulation upregulates ISGs in Huh-7 cells.....	96
3.2.4 HCV blocks IFN- α induced gene expression in Huh-7 cells.....	97
3.2.5 HCV expression significantly inhibits ISRE and GAS induction.....	98
3.2.6 Endogenous ISG expression is dependent on STAT1 and STAT3 expression	100
3.2.7 ISG induction is dependent on STAT1 and STAT3 expression.....	101
3.2.8 STAT3 is required for ISG induction	102
3.2.9 The elongin C binding site is conserved in genotype 1 of HCV.....	103
3.2.10 Orientation of the elongin C binding site in p7.....	104
3.2.11 The E2 protein of HCV has a cul-2 binding site	105
3.2.12 The cul-2 binding site is conserved in genotype 1 of HCV	106
3.2.13 Orientation of the cul-2 binding site	107

3.2.14 HCV NS2 protein has an elongin C binding site	108
3.2.15 The elongin C binding site in NS2 is highly conserved in genotype 1	109
3.2.16 Orientation of the elongin C binding site in NS2.....	110
3.2.17 Cloning of the E2-p7-NS2 region of HCV	111
3.2.18 E2NS2 reduces STAT3 expression.....	112
3.2.19 ENS2 inhibits activation of ISRE and GAS promoter elements.....	113
3.2.20 E2NS2 inhibits ISG induction.....	114
3.2.21 Designing novel peptides against the elongin C binding site in p7	115
3.2.22 An inhibitory peptide against the elongin C binding site in p7 can partially recover STAT3 expression	116
3.2.23 Designing novel peptides against the cul-2 binding site in E2 and the elongin C binding sites in NS2	117
3.2.24 Treatment with a peptide combination restores STAT3 expression.....	118
3.2.25 Treatment with E3 ligase binding site inhibitor peptides blocks HCV-mediated inhibition of ISG induction	119
3.3 Discussion	120
Chapter 4 Anti-viral gene induction in PBMCs from HCV patients	124
4.1 Introduction	124
4.1.1 HCV replication and PBMCs.....	125
4.1.2 PBMCs from HCV patients and STAT proteins.....	127
4.1.3 PBMCs from HCV patients and induction of ISGs	128
4.1.4 Viral genotype and ISG induction in PBMCs	128
4.1.5 PBMCs and response to IFN- α treatment	129
4.1.6 Aims	130
4.2 Results	131
4.2.1 PBMCs upregulate anti-viral gene expression in response to IFN- α	131
4.2.2 PBMCs from HCV patients upregulate ISGs in response to <i>in vitro</i> IFN- α stimulation.....	132
4.2.3 Endogenous expression of anti-viral genes is similar in healthy controls and HCV infected patients	133
4.2.4 ISG induction is similar in patients infected with genotype 1 and those infected with other genotypes	135
4.2.5 Genotype 1 infected patients have similar endogenous ISG expression in their PBMCs compared to genotype non-1 infected patients	137
4.2.6 Patients who failed to respond to therapy have low anti-viral gene induction <i>in vitro</i>	138
4.2.7 Endogenous expression of ISGs is similar in responders and non-responders	140
4.2.8 Difference in induction of ISGs in responders and non-responders is greater in those infected with genotype 1 of HCV	141
4.2.9 IFN- α fails to activate STAT proteins in PBMCs from non-responder patients	142
4.2.10 STAT1 and STAT3 mRNA levels are similar between healthy patients	144
4.2.11 CXCL10 mRNA is elevated in patients who failed to respond to therapy	145
4.2.12 Endogenous mRNA expression levels of Trim 21, but not Trim 19, are elevated in non-responders to therapy	146
4.2.13 There is no significant difference in IFN- α and IFN- β mRNA between responders and non-responders	148
4.2.14 Proteasomal inhibition recovers anti-viral gene expression in non-	

responders	149
4.2.15 STAT3 is required for optimal ISG induction in PBMCs	150
4.2.16 STAT3 is required for the recovery of ISGs in non-responders.....	151
4.3 Discussion	152
Chapter 5 Pre-treatment <i>in vitro</i> responsiveness to IFN- α as a predictor of clinical responsiveness.....	160
5.1 Introduction.....	160
5.1.1 Viral factors.....	160
5.1.2 Host factors	161
5.1.3 HCV, PBMCs and IFN- α therapy	162
5.1.4 Disadvantages of IFN- α /ribavirin therapy	162
5.1.5 Future HCV treatment.....	163
5.1.6 Patient response to treatment	166
5.1.7 Aims	167
5.2 Patient information.....	168
5.3 Results.....	170
5.3.1 Response rates to clinical IFN- α therapy	170
5.3.2 Breakdown of patient response to treatment.....	172
5.3.3 HCV patients (all genotypes) who achieve RVR have similar anti-viral gene expression pre-treatment as patients who do not achieve RVR	174
5.3.4 Genotype non-1 infected HCV patients who achieve RVR have similar pre- treatment ISG expression <i>in vitro</i> as those who fail to demonstrate RVR.....	177
5.3.5 Genotype 1 infected patients who achieve RVR have higher ISG expression in their PBMCs pre-treatment compared to patients who do not have RVR	180
5.3.6 HCV patients (all genotypes) who achieve EVR have higher ISG expression <i>in vitro</i> in their PBMCs pre-treatment	183
5.3.7 Genotype non-1 infected patients who achieve EVR have similar ISG expression in their PBMCs pre-treatment as those who do not achieve EVR.....	186
5.3.8 Genotype 1 infected patients who achieve EVR have higher ISG expression pre-treatment in their PBMCs than patients who do not have EVR	189
5.3.9 ISG expression in pre-treatment PBMCs from patients of all genotypes does not predict who will be PCR –ve at the end of treatment	192
5.3.10 Genotype non-1 infected patients who are PCR +ve at the end of treatment have higher ISG expression in their PBMCs pre-treatment.....	195
5.3.11 Genotype 1 infected patients who are PCR –ve at the end of treatment have higher ISG expression in their PBMCs pre-treatment	198
5.3.12. Patients (all genotypes) who achieve an SVR tend to have higher ISG expression in their PBMCs pre-treatment.....	201
5.3.13. There is no correlation with pre-treatment ISGs in PBMCs and SVR in genotype non-1 HCV patients.....	204
5.3.14 Genotype 1 infected patients who achieve SVR have significantly higher ISG expression pre-treatment.....	208
5.3.15 Genotype 1 infected patients who will respond to IFN- α therapy can be identified pre-treatment through analysis of PBMC responsiveness to IFN- α stimulation.....	212
5.3.16 Patients of all genotypes who relapse have low <i>in vitro</i> ISG expression pre- treatment.....	215
5.3.17 Genotype non-1 infected patients who relapse have low ISG expression pre- treatment.....	218

5.3.18 Genotype 1 infected patients who relapse have low ISG induction pre-treatment.....221

5.3.19 CXCL10 expression pre-treatment is similar in genotype 1 infected patients who have an SVR and those who do not224

5.4 Discussion225

Chapter 6: Final Discussion232

6.1 Future work240

References242

List of Figures

Figure 1.1. Prevalence of HCV worldwide24

Figure 1.2. The HCV polyprotein27

Figure 1.3. HCV virion structure29

Figure 1.4. Frameshift mutation in core result in F protein translation30

Figure 1.5. Receptors that mediate HCV internalisation33

Figure 1.6. Cases of HCV confirmed in Ireland by the National Virus Reference Lab
between 1989-200437

Figure 1.7. TLR receptor activation upregulates type I IFNs and pro-inflammatory
cytokines43

Figure 1.8. Cytosolic RLRs activation promotes type I IFN production44

Figure 1.9. IFN- α signals through the JAK-STAT pathway51

Figure 1.10. Structure of JAK kinases52

Figure 1.11. Overview of STAT protein structure53

Figure 1.12. HCV NS3-4A and NS5A block TLR signal transduction57

Figure 1.13. HCV NS3-4A inhibits RLR-mediated activation of Type I IFNs.....59

Figure 1.14. Overview of HCV NS5A domains that interact with PKR and OAS.....61

Figure 2.1. RNA yield from PBMCs with or without IFN- α stimulation73

Figure 2.2. RNA quality: healthy PBMCs73

Figure 2.3. RNA quality: PBMCs from HCV patients74

Figure 2.4. GeNorm analysis of reference genes75

Figure 3.1. Proteasomal inhibition recovers STAT3 expression in PBMCs from HCV
patients89

Figure 3.2. Polyubiquitination of target proteins results in protein degradation via the
proteasome90

Figure 3.3. The elongin C binding site in RSV NSI, HCV p7 and VHL92

Figure 3.4. Proposed model for HCV-mediated STAT proteins degradation.....92

Figure 3.5. STAT1 and STAT3 protein expression is reduced in Huh-7 cells expressing
HCV94

Figure 3.6. HCV expression does not reduce STAT1, STAT2 and STAT3 mRNA
expression.....95

Figure 3.7. IFN- α upregulates ISG expression in Huh-7 hepatocytes96

Figure 3.8. HCV blocks ISG induction in Huh-7 hepatocytes.....97

Figure 3.9. HCV inhibits ISRE and GAS promoter activity.....99

Figure 3.10. Endogenous ISG expression is dependent on STAT1 and STAT3 expression
.....100

Figure 3.11. ISG induction is dependent on STAT1 and STAT3101

Figure 3.12. STAT3 siRNA reduces ISG induction in Huh-7 hepatocytes102

Figure 3.13. The Elongin C binding site in different HCV genotypes.....103

Figure 3.14. Helical wheel projection of the elongin C binding site104

Figure 3.15. The cul-2 binding site of HCV105

Figure 3.16. The cul-2 binding site in different HCV genotypes.....106

Figure 3.17. Helical wheel projection of the cul-2 binding site.....107

Figure 3.18. The elongin C binding site in NS2108

Figure 3.19. The elongin C binding site is conserved in genotype 1 of HCV109

Figure 3.20. The orientation of the elongin C binding site in NS2.....110

Figure 3.21. Expression of E2NS2 in Huh-7 hepatocytes111

(B)112

Figure 3.22. E2NS2 inhibits expression of STAT3112

Figure 3.23. E2NS2 is an effective inhibitor of ISRE and GAS luciferase induction ..113

Figure 3.24. E2NS2 inhibits induction of PKR and MxA114

Figure 3.25. Analysis of STAT3 protein expression in the presence of inhibitory peptide116

Figure 3.26. Treatment with a combination of inhibitory peptides restores STAT3 protein expression118

Figure 3.27. Treatment with inhibitory peptides can block HCV inhibition of ISG induction.....119

Figure 4.1. HCV replication involves synthesis of negative and positive strands of the viral genome.....124

Figure 4.2. HCV NS2 is expressed in PBMCs from HCV infected patients126

Figure 4.3. STAT1 and STAT3 are absent in the PBMCs of HCV infected patients127

Figure 4.4. qRT-PCR analysis of ISG induction in PBMCs from healthy individuals.131

Figure 4.5. qRT-PCR analysis of IFN- α induced ISGs in PBMCs from healthy and HCV +ve patients132

Figure 4.6. qRT-PCR analysis of endogenous ISGs levels in PBMCs from healthy and HCV individuals134

Figure 4.7. ISG induction in PBMCs from HCV patients infected with genotype 1 of the virus and other genotypes136

Figure 4.8. Genotype analysis of endogenous ISG levels in PBMCs from HCV infected patients137

Figure 4.9. ISG induction in PBMCs from HCV +ve patients who failed to respond to therapy.....139

Figure 4.10. Endogenous ISG levels in responders and non-responders to therapy.....140

Figure 4.11. ISG induction in genotype 1 infected patients who failed to respond to therapy.....141

Figure 4.12. Flow cytometric analysis of pSTAT1 and pSTAT3 expression in response to IFN- α143

Figure 4.13. STAT1 and STAT3 mRNA expression is similar in PBMCs from healthy controls and HCV infected patients144

Figure 4.14. Endogenous CXCL10 and CXCL10 induction in PBMCs from responders and non-responders analysed by qRT-PCR.....145

Figure 4.15. Expression of Trim 21 and Trim 19 mRNA in PBMCs from responders and non-responders147

Figure 4.16. IFN- α and IFN- β mRNA expression in PBMCs of non-responders analysed by qRT-PCR148

Figure 4.17. Proteasomal inhibition recovers ISG induction in PBMCs.....149

Figure 4.18. STAT3 is required for optimal ISG induction in PBMCs.....150

Figure 4.19. STAT3 is required for the recovery of ISGs via the proteasome in non-responder patients151

Figure 5.1. HCV infected patients (all genotypes) who achieve RVR have similar ISG expression pre-treatment as patients who fail to achieve RVR.....175

Figure 5.2. Genotype non-1 infected patients who achieve RVR have similar ISG expression pre-treatment as patients who fail to achieve RVR.....178

Figure 5.3. Genotype 1 patients who achieve RVR have higher pre-treatment ISG

expression than patients who fail to achieve RVR.....	181
Figure 5.4. HCV infected patients (all genotypes) who achieve EVR have similar ISG expression pre-treatment as patients who fail to achieve EVR.....	184
Figure 5.5. Genotype non-1 infected patients who achieve EVR have similar ISG expression pre-treatment as patients who fail to achieve EVR.....	187
Figure 5.6. Genotype 1 patients who achieve EVR have higher pre-treatment ISG expression than patients who fail to achieve EVR.....	190
Figure 5.7. HCV infected patients (all genotypes) who are PCR –ve and PCR +ve at the end of treatment have similar ISG expression pre-treatment.....	193
Figure 5.8. Genotype non-1 infected patients who are PCR –ve or PCR +ve at the end of treatment have similar ISG expression pre-treatment	196
Figure 5.9. Genotype 1 patients who are PCR –ve at the end of treatment have higher pre-treatment ISG expression than patients who remain HCV +ve.....	199
Figure 5.10. HCV infected patients (all genotypes) who achieve SVR have similar ISG expression pre-treatment as patients who fail to achieve SVR.....	202
Figure 5.11. Genotype non-1 infected patients who achieve SVR have similar ISG expression pre-treatment as patients who fail to achieve SVR.....	205
Figure 5.12. Genotype 1 patients who achieve SVR have significantly higher pre-treatment ISG expression than patients who fail to achieve SVR	209
Figure 5.13. Patients with SVR have significant induction of ISGs pre-treatment	213
Figure 5.14. Patients (all genotypes) who relapse after treatment have lower ISG expression pre-treatment	216
Figure 5.15. There is no significant difference in ISG expression in patients infected with genotype non-1 who relapse and those who achieve SVR	219
Figure 5.16. Genotype 1 infected patients who relapse have lower ISG expression pre-treatment.....	222
Figure 5.17. CXCL10 mRNA expression is similar in genotype 1 infected patients who achieve SVR and those who do not	224
Figure 6.1. E2NS2 is a viral E3 ligase	234
Figure 6.2. HCV degrades STAT proteins via the proteasome to block induction of anti-viral genes	240

List of Tables

Table 1.1. Members of the IFN family.....46

Table 1.2. Type I IFNs upregulate 100s of genes with diverse functions49

Table 2.2. List of peptides synthesised71

Table 2.2. Primers for qRT-PCR76

Table 2.3. Antibodies used for western blotting.....78

Table 3.1 Peptides designed to block HCV inhibition of IFN- α signalling115

Table 3.2. Peptide sequences for the peptides designed against the cul-2 binding site in E2 and the elongin C binding site in NS2117

Table 5.3. On-treatment response to IFN- α and ribavirin therapy167

Table 5.4. Summary of patient characteristics169

Table 5.3. Number of patients who achieved RVR, EVR, EOT and SVR.....171

Table 5.4 Breakdown of response rates.....173

Table 5.5. Summary of ISG expression and RVR: all genotypes176

Table 5.6. Summary of ISG expression and RVR: genotype non-1179

Table 5.7. Summary of ISG expression and RVR: genotype 1182

Table 5.8. Summary of ISG expression and EVR: all genotypes185

Table 5.9. Summary of ISG expression and EVR: genotype non-1188

Table 5.10. Summary of ISG expression and EVR: genotype 1191

Table 5.11. Summary of ISG expression and EOT: all genotypes.....194

Table 5.12. Summary of ISG expression and EOT: genotype non-1197

Table 5.13. Summary of ISG expression and EOT: genotype 1200

Table 5.14. Summary of ISG expression and SVR: all genotypes203

Table 5.15. Summary of ISG expression and SVR: genotype non-1206

Table 5.16. Summary of pre-treatment ISG expression in PBMCs from genotype non-1 infected patients207

Table 5.17. Summary of ISG expression and SVR: genotype 1210

Table 5.18. Summary of pre-treatment ISG expression in PBMCs from genotype 1 infected patients211

Table 5.19. Cut-off gene expression values for predictive values for achieving SVR .214

Table 5.20. Cut-off gene expression values for predictive values for not achieving SVR214

Table 5.21. Summary of ISG expression and relapse: all genotypes.....217

Table 5.22. Summary of ISG expression and relapse: genotype non-1220

Table 5.23. Summary of ISG expression and relapse: genotype 1223

Acknowledgements

There are so many people I would like to thank who have helped and encouraged me throughout the past three years.

Firstly, I have to express my sincere gratitude to and admiration for Prof. Cliona O'Farrelly. Cliona's love of immunology and all things scientific is infectious and I could not have done this thesis or grew as a scientist without her guidance and her support. I also have to express my extreme gratitude to Dr. Nigel Stevenson, who has helped and guided me throughout this entire process, always full of encouragement. I think my motto for the rest of my scientific career shall be "what would Nigel do?!" Another inspiring individual who deserves a massive acknowledgement is Dr. Andrew Lloyd. I am forever grateful for his 'significant' help throughout the PhD and in writing this manuscript.

I cannot begin to articulate how thankful I am to the entire comparative immunology groups, members both past and present, for all their friendship and support. My PhD would have been a very different and far less enjoyable experience without them. In particular I am so grateful to Aideen Collins, who started with me on day one and we shared every moment of this journey together. I am so lucky that I have had such an amazing friend to do a PhD with. When I started this, I expected a PhD when I finished and what I have gotten is a PhD (hopefully!) and a friend for life. I have to 'profoundly' thank my proof-readers, chocolate pushers and tea buddies in the office. My neighbour and wonderful friend Aoife Kelly (I am forever indebted to this lambda expert extraordinaire!), my fellow Kidare- and Christy Moore lover- Cormac Whelahan, Ronan Fahey (and his willy-nilly ways), the always encouraging Sarah Whelan (aka Sarahair), peptide expert Tatyana Lysakova-Devine and my fellow pre-treatment torch-bearer Mary O'Neill. I also want to say a massive and special thanks to both Catherine Keogh and Fernando Narciandi, who have helped me from the moment I started this PhD. Past members of the lab I want to particularly acknowledge are Ronan O'Shaughnessy, Liz Ryan, Aspininas Chapwanya and Shahzad Sharwar.

I feel honoured that I got to do my PhD in the School of Biochemistry and Immunology in TCD, an amazing department filled with the most wonderful and

inspiring people. I particularly want to thank the O'Neill and Bowie labs, Liam Cross, Noel Breslin, Miriam Wilson and last but in no means least, Mary Pat O'Sullivan, who has helped me not just through my PhD but my degree as well. The support from St. Vincent's University Hospital has also been fantastic and I would like to thank Prof. John Hegarty and all the nurses and doctors who have helped with patient samples, particularly Carol McNulty for all her assistance.

I would like to thank all my friends for their love and support, a session in Berla's is most defiantly in order! I particularly want to thank Niamh McClelland with her never-ever ending ability for making me happy through hilarious stories, a supply of markers and inspirational cups. I really could not have done this without Robert Roche and his unwavering support, despite his best efforts to corrupt my PhD with the stupid portal! The last three years would have been much harder without him and I shall be forever grateful. Finally, I have to thank my amazing family. In particular, my much-loved grandparents, my fantastic brothers James and Liam, who never fail to put me in a good mood, and my wonderful parents. I could never have done this, or anything, without the support, encouragement and love from my Mam and Dad and I will be forever grateful for everything they have done, and continue to do, for me.

Publications and funding

Stevenson NJ* & Bourke NM*, Keogh CA, Lloyd AT, Ryan EJ, Binder M, Elliott J, Keating S, Fanning L, Bowie AG, Johnston JA, Hegarty JE, Long A, O'Farrelly C: HCV induced STAT3 proteasomal degradation blocks IFN- α anti-viral responses: A novel immune evasion mechanism. In preparation for **Hepatology**.

***Joint first authors**

Bourke NM, Sarwar S, Norris S, Stewart S, Hegarty JE, Stevenson NJ, O'Farrelly C: *In vitro* response of PBMCs to IFN- α predicts Hepatitis C Virus (HCV) genotype 1 infected patients who will not require additional protease inhibitors. In preparation for **Hepatology**.

All work was funded by Science Foundation Ireland.

Abbreviations

AIM2	Absent in melanoma 2
ALT	Alanine transaminase
APC	Antigen presenting cell
ARFP	Alternate reading frame protein
AST	Aspartate aminotransferase
AUC	Area under the curve
B2M	β 2 microglobulin
BCA	Bicinchoninic acid
BCR	B cell receptor
BSA	Bovine serum albumin
cDC	Conventional DC
CI	Confidence intervals
CLN1	Claudin N1
COX	Cyclooxygenase
CTL	Cytotoxic T lymphocyte
Cul	Cullin
CXCL10	Chemokine C-X-C motif chemokine 10
DC	Dendritic cell
dH ₂ O	Distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2NS2	E2-p7-NS2
E. Coli	Escherichia coli

EDTA	Ethylenediaminetetraacetic acid
eIF2- α	Eukaryotic initiation factor 2- α
EOT	End of treatment
ER	Endoplasmic reticulum
EV	Empty vector
EVR	Early virological responder
FCS	Foetal calf serum
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	γ -activated sequence
GTP	Guanosine-5'-triphosphate
GUSB	β -glucuronidase
GWAS	Genome-wide association studies
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDL	High-density lipoprotein
HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HVR	Hypervariable region
HSV	Herpes simplex virus
IFN- α	Interferon-alpha
IFNAR	IFN- α receptor
IKK	I κ B kinase
IL	Interleukin
IP-10	IFN γ -induced protein

IPS-1	IFN promoter stimulator-1
IRAK	IL-1 receptor-associated kinase
IRES	Internal ribosome entry site
IRFs	IFN-regulatory factors
ISDR	IFN sensitivity determining region
ISGs	IFN stimulated genes
ISGF-3	IFN stimulated gene factor
ISRE	IFN stimulated response element
IVDA	Intravenous drug abuse
JAK	Janus kinase
KO	Knock-out
LB	Luria-Bertani
LDLR	Low-density lipoprotein receptor
LGP-2	Laboratory and of genetics and physiology-2
JH	JAK-homology
MAL	MyD88 adapter like protein
MAPK	Mitogen-activated protein kinase
MDA-5	Melanoma differentiation factor-5
Min	Minute
MxA	Myxovirus A
MyD88	Myeloid differentiation primary response gene (88)
NCR	Non-coding region
NF	Nuclease-free
NF-κB	Nuclear factor-κB
NK	Natural killer
NLRs	NOD-like receptors

NOD	Nucleotide binding and oligomerization domain
NPV	Negative predictive value
NS	Non-structural
OAS	2'5'-oligoadenylate synthetase
ORF	Open reading frame
PAMPs	Pattern associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PEG	Pegylated
PHYIN	Pyrin and HIN domain-containing protein
PIAS	Protein inhibitors of STAT
PKR	Double-stranded RNA-dependent protein kinase R
PMSF	Phenylmethanesulfonylfluoride
pp	Pseudo-particle
PP2A	Protein phosphatase 2
PPIA	Peptidylprolyl isomerase A
PPV	Positive predictive value
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time RT-PCR
RdRp	RNA-dependent RNA polymerase
RIG	Retinoic acid-inducible gene 1
RIN	RNA integrity number
RLRs	RIG-like receptors

RNA	Ribonucleic acid
ROC	Receiver operating characteristic curve
rpm	Revolutions per minute
RVR	Rapid virological responder
RIN	RNA Integrity Number
RIPA	Radioimmunoprecipitation
RPMI	Roswell Park Memorial Institute
RPS15	Ribosomal protein 15
RSV	Respiratory syncytial virus
SARM	Sterile- α and armadillo motif containing protein
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SF	Serum-free
shRNA	Short interfering RNA
siRNA	Small interfering RNA
SJH	St. James's Hospital
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signalling
SR-B1	Scavenger receptor class B type 1
STAT	Signal Transducers and Activators of Transcription
SVR	Sustained virological responder
SVUH	St. Vincent's University Hospital
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TEM	Tetraspanin-enriched microdomains

TIR	Toll/IL-1 receptor
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
TRAM	Trif-related adapter molecule
TRIF	Toll–IL-1 receptor domain–containing adapter inducing IFN- β
Trim	Tripartite motif-containing protein
Tris	Tris(hydroxymethyl)aminomethane
Tyk	Tyrosine-protein kinase
VACV	Vaccinia virus
VHL	Von Hippel-Lindau
WCC	Whole cell count
WNV	West Nile virus
WT	Wild-type

Abstract

Successful anti-viral immunity is dependent on effective activation of the innate immune response. The majority of individuals infected with hepatitis C virus (HCV) fail to eliminate the virus and progress to chronic infection. Treatment for HCV is with the anti-viral cytokine interferon-alpha (IFN- α), a potent activator of the innate immune system. Treatment response rates vary depending on viral genotype, with genotype 1 infected patients having response rates of less than 50%. IFN- α signals through the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway, specifically activating STAT1, STAT2 and STAT3, which induces many IFN stimulated genes (ISGs). Studies from our group showed that STAT1 and STAT3 expression is reduced in peripheral blood mononuclear cells (PBMCs) from HCV patients. Therefore, our central hypothesis was that HCV directly inhibits IFN- α signalling, thus blocking anti-viral immune responses. We also proposed that *in vitro* expression of IFN- α induced ISGs would predict clinical responsiveness.

We found that HCV targeted STAT3 to inhibit induction of ISGs, such as for proteins such as protein kinase R (PKR), 2'5'- oligoadenylate synthetase (OAS) and myxovirus resistance A (MxA), revealing a novel role for STAT3 in IFN- α activated innate immune responses. Proteasomal inhibition restored ISG induction in PBMCs from patients who were non-responsive to IFN- α stimulation in a STAT3-dependent manner. Bioinformatic analysis was carried out to identify functionally relevant conserved regions of the HCV genome, particularly viral genotype 1, that allow HCV to hi-jack host proteasome degradation pathways and target STAT3. Using small peptide inhibitors against these conserved regions, both STAT3 expression and ISG induction was recovered.

Patients who had failed to respond to therapy had low ISG induction in their PBMCs *in vitro* following IFN- α stimulation. We therefore analysed *in vitro* response to IFN- α in PBMCs from patients commencing IFN- α therapy and correlated ISG induction with therapeutic response. Quantification of PKR, OAS and MxA expression *in vitro* following IFN-stimulation prior to treatment reliably predicted genotype 1 infected patients who would achieve viral clearance at the end of therapy.

In conclusion, this research found that HCV specifically targets STAT3 to evade anti-viral immune responses and this inhibition could be reversed by targeting

conserved regions of the viral genome responsible for mediating protein degradation. Having observed this profound effect within patient PBMCs, we were able to use PBMCs to accurately identify which genotype 1 infected patients would respond to IFN- α therapy prior to treatment. Our findings present rational targets within the HCV genome for the development of novel therapeutics and provide a clinical predictive assay to determine patient response to IFN- α treatment.

Chapter 1

Introduction

Chapter 1 Introduction

HCV is a bloodborne virus estimated to currently infect 159 million people worldwide. Epidemiological data suggests that approximately 400,000 individuals are chronically infected in Australia and Oceania, 14 million in the Americas, 16 million in the Middle East, 17.5 million in Europe, 28.1 million in Africa and 83 million in Asia (Fig. 1.1) [1]. Precise data are unknown as initial HCV infection is often asymptomatic, but estimated figures indicate that HCV is a significant global health burden. Chronic infection over decades can lead to the significant damage of the liver and the development of serious complications including cirrhosis, hepatocellular carcinoma and eventual liver failure. HCV infection is the leading cause of liver transplantation in the developed world and the World Health Organisation believe that 350,000 people worldwide die every year from HCV related illnesses.

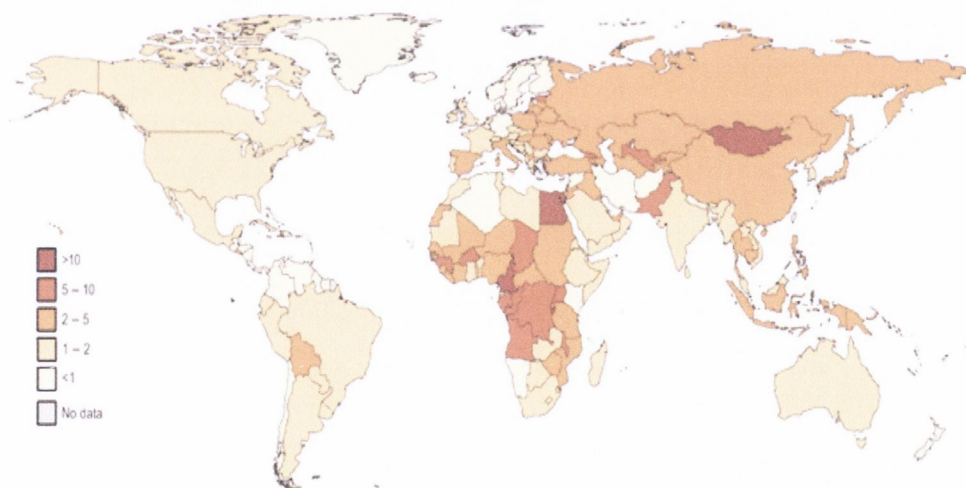


Figure 1.1. Prevalence of HCV worldwide

HCV prevalence is highest in Asia and Africa, with Egypt (14%), Cameroon (13.8%), Burundi (11.3%) and Mongolia (10.7%) having the highest infection rates worldwide. Countries in Europe, particularly within Eastern Europe also have a significant proportion of the population infected. Adapted from [1].

Viral infection is usually rapidly detected and eliminated by robust anti-viral defences within the host immune system. However, in individuals infected with HCV, 50-90% of those infected fail to eliminate the virus and go on to develop chronic HCV infection [2], suggesting that HCV has evolved mechanisms of overcoming the host immune response. IFN- α proteins are a family of 13 cytokines produced by many cells upon viral infection and exert potent anti-viral activity through upregulation of over 500 genes associated with effective viral clearance [3]. The standard therapy for HCV is a combination of IFN- α with the nucleoside analogue ribavirin. IFN- α therapy is an expensive, arduous treatment regime which causes serious side effects such as depression and anaemia. The high cost of treatment eliminates the opportunity of therapy for many in the developing and third world, who comprise the majority of those infected worldwide. The potent activation of anti-viral responses by IFN- α suggests that therapeutic administration of IFN- α to HCV infected individuals would lead to the successful eradication of the virus. However, this therapy has suboptimal response rates; viral clearance after therapy can be as low as 40-50% depending on viral genotype. Therefore, due to the high cost of treatment, severe side effects and poor success rate, it is critical that patients who will ultimately fail to respond are identified prior to commencing therapy thus avoiding unnecessary treatment. Several viral and host factors have been implicated as predictors of therapeutic response to IFN- α , including viral genotype [4], innate immune genes, such as polymorphisms near IFN- λ genes [5], and hepatic ISG expression [6]; however as yet, there is no pre-treatment assay to determine who will respond and who will not.

Furthermore, as HCV interferes with the action of IFN- α , it is crucial that the molecular mechanisms used by HCV to block the action of IFN- α are thoroughly understood. Fully elucidating the interaction between HCV and the innate immune system would enhance the development of novel anti-viral therapies.

1.1 Hepatitis C Virus

HCV was formally identified in 1989 after a decade long search to discover the causative agent of non-A, non-B hepatitis [7]. HCV is an RNA virus from the Hepacivirus genus within the Flaviviridae family, a family that also includes Flaviviruses and Pestiviruses. There are seven genotypes of HCV, which can have as much as 33% nucleotide sequence diversity between genotype. Each genotype is further separated into many subtypes and quasispecies [8]. HCV has a positive single-stranded genome, ~9,600 nucleotides in length, and encodes 10 viral proteins. A genome of 9,600bp is an extremely small viral genome, as some DNA viruses can be as large as 300 to 1,200 kbs and encode for 100s of viral proteins [9]. The primary site of HCV replication is the liver and it is estimated that a single infected hepatocyte produces 10 to 100 viral particles per day [10]. The viral genome is comprised of a 5' non-coding region (NCR), a long open reading frame (ORF) and a 3' NCR. The ORF is both co- and post-translationally modified to produce viral proteins (Fig.1.2). The 5' NCR is highly conserved across genotypes and contains the internal ribosome entry site (IRES), which is involved in cap-independent translation of RNA. The functions of HCV proteins have been the subject of intense scrutiny in an effort to identify novel therapeutic targets. Although many roles have been discovered, the function of several of these proteins still remains unclear. Structural proteins of HCV include core and the envelope glycoproteins E1 and E2. The non-structural (NS) proteins consist of p7, NS2, NS3, NS4A, NS4B, NS5A and the NS5B RNA-dependent RNA polymerase (RdRp) [11].

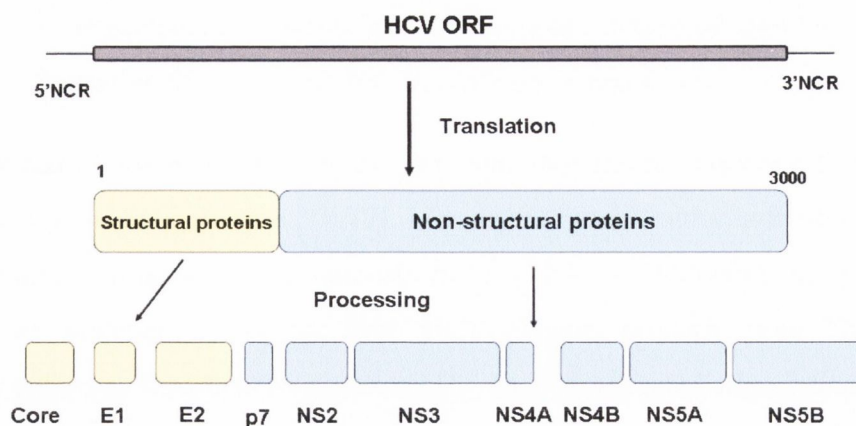


Figure 1.2. The HCV polyprotein

The HCV open reading frame (ORF), between the 5' non-coding region (NCR) and 3'NCR, is translated into the HCV polyprotein. The ~3000 amino acid polyprotein is made up of structural and NS proteins. The polyprotein is post-translationally cleaved by host peptidases and viral proteases to produce 10 viral proteins. The structural proteins include core, E1 and E2 and the non-structural proteins include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.

1.1.1 Structural proteins

HCV structural proteins are expressed in the amino terminus of the polyprotein and they assemble to form the viral particle. The core protein of HCV is an RNA binding protein that has been shown to play a pivotal role in the assembly of the viral nucleocapsid. Core directs assembly of viral NS proteins to lipid droplets on the endoplasmic reticulum (ER), where the NS proteins instigate viral replication [12]. Core has also been implicated in host cellular processes including apoptosis, lipid metabolism, and carcinogenesis [13]. The role of core in apoptosis remains controversial, with many studies claiming that core enhances apoptosis and many others concluding it protects against programmed cell death. For example, one study found that core enhanced apoptosis in the Huh-7 hepatocyte cell line through caspase activation and cytochrome C release from the mitochondria [14]. In contrast, *in vivo* studies found that transgenic mice expressing core, E1, E2 and NS2 were resistant to cytochrome c release from the mitochondria and had less caspase 3 and caspase 9 activation [15]. Conflicting reports about the effect of core on apoptosis are most likely

due to different experimental systems and the complex nature of apoptotic pathways within cells, illustrating that the exact function of core is unknown.

Core has also been reported to exhibit some immunomodulatory effects, such as inducing an inflammatory profile [16,17]. Monocytes from healthy individuals produce the inflammatory cytokine tumour necrosis factor (TNF) - α following stimulation with core proteins; however, monocytes from HCV patients produce more TNF- α upon stimulation with core than healthy controls [18]. Indeed, repeated stimulation with core failed to induce tolerance in monocytes from HCV patients, who remained hyper-responsive to core stimulation, unlike healthy controls that produced less TNF- α over time following repeated stimulation with core protein. This indicates that healthy monocytes become tolerant, preventing excessive inflammation. This is an interesting observation as persistent inflammation within the liver during HCV infection is the cause of hepatic complications associated with the virus. Triggering of TNF- α , interleukin (IL) -1 and IL-10 production by monocytes stimulated with core, as well as a lack of IL-12 production, blocks dendritic cell (DC) differentiation, which are key immune cells responsible for mediating innate and adaptive immune responses [16]. Conversely, core expression in HeLa and hepatoma cells strongly inhibited expression of the pro-inflammatory cellular enzyme cyclooxygenase 2 (Cox-2) [19]. Difference in the effects of core on the inflammatory response is most likely due to the difference in experimental cell types and may reflect mechanisms used by HCV to balance the immunomodulatory effect it has on the immune system and avoid detrimental inflammatory destruction of the host.

The envelope glycoproteins, E1 and E2, form the viral envelope and are modified by N-linked glycans. Both are cleaved by host signal peptidase in the ER and, as an E1-E2 heterodimer, play a pivotal role in HCV viral entry, replication and virion assembly [20] (Fig. 1.3).

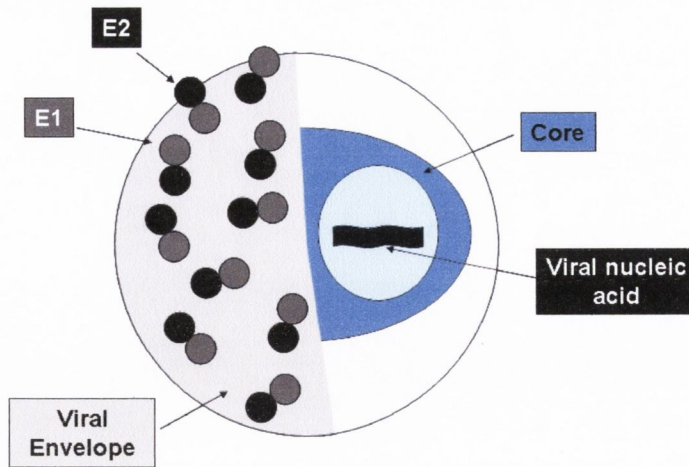


Figure 1.3. HCV virion structure

The HCV proteins E1 and E2 form heterodimers which, along with host proteins, form the viral envelope of the viral particle. Within the virion, the viral nucleic acid is surrounded by core protein.

As E1 and E2 form the viral envelope, and therefore are exposed to the extracellular humoral immune system, they are frequently the candidate antigens used in the efforts to develop a HCV vaccine. Patients who spontaneously clear HCV infection have significantly higher serum levels of broadly neutralising antibodies that recognise epitopes in E1 and E2, compared to patients who do not [21]. In fact, patients who spontaneously resolve HCV infection have been shown to be rapid inducers of neutralising antibodies against the E1-E2 heterodimer whereas those with chronic infection showed low titres of antibodies during the acute stage of infection [22]. Vaccine development has primarily focused on raising antibodies against E1E2 or E2 alone, with little success. However, a recent study found that chimpanzees vaccinated against E1 induced broadly neutralising antibodies and cleared subsequent viral challenge, compared to those vaccinated against E2 (without the hyper-variable region (HVR) -1) or unvaccinated chimps [23]. Further understanding of the innate immune response to these two proteins, and the subsequent induction of effective adaptive immune responses, is needed for the development of new therapeutic vaccines against HCV.

A more elusive structural protein sometimes found during HCV infection results from a +1 ribosomal frameshift mutation within core that produces an alternative reading frame protein (ARFP) or F protein [24]. This phenomenon has been observed in genotype 1a and 1b of HCV; genotype 1a F protein occurs as a result of a +1 frameshift at codon 11 of core; genotype 1b F protein occurs following a frameshift mutation at codon 42 of core (Fig.1.4). Although the role of the F protein in the viral life cycle is still undefined, patient antibodies against the F protein are negatively correlated with HCV viral titres, and T lymphocytes against the F protein exist in some HCV infected patients [25,26]. The exact function of this protein remains to be defined.

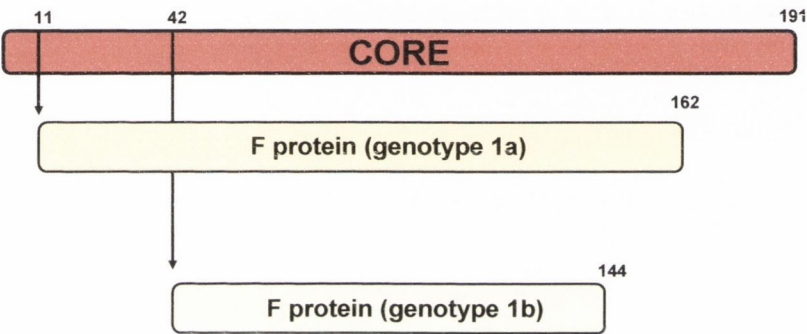


Figure 1.4. Frameshift mutation in core result in F protein translation

A frameshift mutation at codon 11 in HCV genotype 1a results in the production of an F protein of 151 amino acids in length. A frameshift mutation at codon 42 in HCV genotype 1b results in the production of an F protein of 102 amino acids in length.

1.1.2 Non-structural proteins

NS proteins of HCV have many diverse roles in the viral life cycle, including polypeptide cleavage and immunomodulatory roles. p7 is the smallest viral protein and assembles into hexamers, that form an ion channel in the ER membrane [27]. Formation and insertion of p7 hexamers into membranes regulates proton conductance in vesicles to reduce acidification, an event crucial for the production of infectious viral particles [28]. The p7 protein is hypothesised to be an important virulence factor, involved in the

late stage of HCV replication, based on the observation that p7 is essential for infectivity of HCV in chimpanzees [29]. This indicates an important role for p7 in viral life cycle. This small protein, so critical for viral vitality, requires much more research to uncover its complete function.

NS2-3 is a cysteine autoprotease that cleaves the HCV polypeptide between NS2 and NS3A. NS2-3 is needed to establish replicating HCV infection in chimpanzees [30]. Cleaved NS2 protein has been implicated in the inhibition of gene synthesis and induction of apoptosis within cells [31]. NS2 also exerts effects on cell growth by inducing cell cycle arrest in the S phase through down-regulation of cyclin A expression, an effect demonstrated in both hepatic and non-hepatic cell lines [32]. Recently, NS2 has been shown to have a positive effect on HCV translation by upregulating HCV IRES-dependent translation in Huh-7 cells and inhibiting NS5B RdRp activity in Huh-7, HeLa and HepG2 cells, thus shifting the balance from RNA replication to RNA translation [33].

The NS3 protein contains two domains, an N-terminal serine-type protease domain and a C-terminal helicase/NTPase domain and has important functions in the viral life cycle and interaction with the immune system, making it an attractive target for novel drug design. The protease function of NS3 is enhanced when it non-covalently associates with its cofactor NS4A, and this complex has roles in both processing the NS polypeptide and in viral replication. The NS3-4A protease cleaves the HCV polypeptide between NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. Stable NS3 expression is dependent on NS4A as deletion of NS4A results in the rapid degradation of NS3 within the cytosol. Both proteins are essential for HCV viability [34,35]. The NS3-4A protease has immunomodulatory effects on innate immunity, as discussed in section 1.6.

NS4B is a hydrophobic viral protein that induces the formation of membranous structures on the ER necessary to support HCV RNA replication [13]. NS4B also has an important nucleotide binding domain, which mediates the binding and hydrolysis of guanosine-5'-triphosphate (GTP), a substrate for RNA synthesis during transcription [36]. Mutation of amino acids within this domain inhibits viral replication, highlighting NS4B as a future target for HCV anti-viral drugs.

NS5A is a phosphoprotein which can be found in both phosphorylated and hyperphosphorylated forms. The phosphorylation of NS5A is conserved among Hepacivirus and Pestiviruses, demonstrating its importance in the Flaviviridae family life cycle [11]. NS5A has profound effects on the innate immune system (discussed in section 1.6) and can modulate host cell cycle control to propagate viral replication. NS5A directly targets many mitogen-activated protein kinase (MAPK) signalling pathways which allows it to control the cell growth cycle, presumably to benefit viral replication [37]. Interestingly, mutations in the IFN sensitivity determining region (ISDR) of NS5A have been linked with response to IFN- α treatment [38,39].

NS5B protein is an RNA-dependent RNA polymerase, making it a critical enzyme for HCV viral replication and an attractive target for direct anti-viral drug development. NS5B uses the positive strand RNA genome of HCV as a template and initiates synthesis of a complimentary negative RNA strand by binding to the 3'UTR. Mutations in motifs within NS5B that mediate its RdRp function dramatically block viral replication [40]. Other functions attributed to NS5B include binding to and inhibiting the anti-viral GTPase guanylate binding protein 1 and inhibiting TNF- α stimulated nuclear factor κ B (NF- κ B) activation, which is necessary for the induction of a pro-inflammatory immune response [41,42].

As HCV encodes such a small number of proteins, it is not surprising that these proteins are multifunctional and often work synergistically to promote virus survival and propagation. An example of this is NS5B, whose expression results in upregulation of the anti-viral cytokine IFN- β . Co-expression of NS4A, NS4B and NS5A, counteract this activation of IFN- β , possibly reflecting strategies evolved by the virus to limit its own activation of the immune system [43]. NS4A can bind to NS3, inhibiting the enzymatic activity of the NS2-3 protease to possibly control proteolytic cleavage events during replication [44]. These two examples illustrate the importance of studying HCV proteins in the context of one another and that studies of one protein alone cannot draw definite conclusions as to that protein's function.

1.1.3 HCV viral life cycle

The HCV viral life cycle commences with HCV mediating its entry into a host cell. The membrane of the HCV virion is made of host-derived proteins and E1-E2 heterodimers. These heterodimers bind several defined receptors including CD81, scavenger receptor class B type 1 (SR-B1), claudin N1 (CLN1), occludin, glycosaminoglycans (GAGs), low-density lipoprotein receptor (LDLR) and the mannose binding lectins, DC-SIGN and L-SIGN [45] (Fig. 1.5).

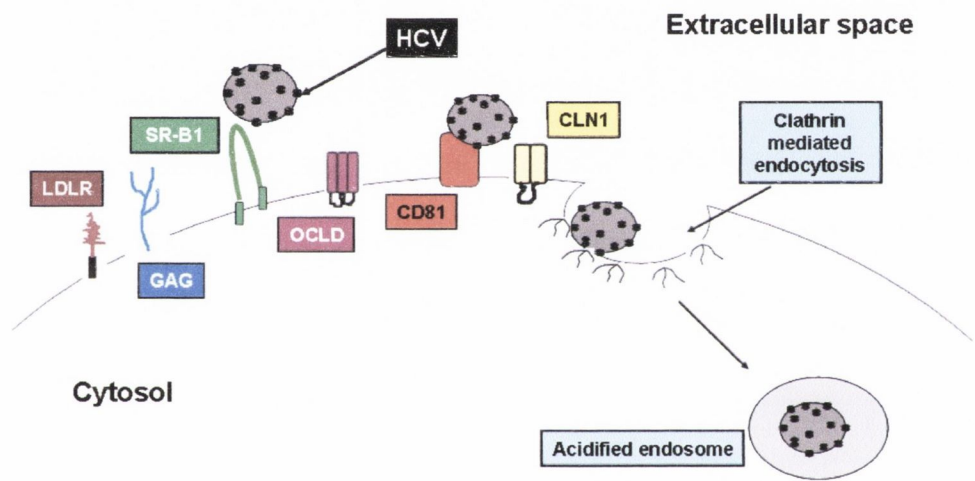


Figure 1.5. Receptors that mediate HCV internalisation

Several receptors on the cell membrane can bind to and facilitate HCV internalisation into the cell, including low-density lipoprotein receptor (LDLR), glycosaminoglycans (GAGs), scavenger receptor class B type 1 (SR-B1), occludin (OCLD), CD81 and claudin N1 (CLN1). Following binding to the receptor, HCV is internalised via clathrin-mediated endocytosis and the virus is internalised in the cytosol in an acidified endosome.

The first identified HCV receptor was CD81, a type III membrane glycoprotein that can form tetraspanin enriched microdomains (TEMs) at the cell membrane, similar to the formation of lipid rafts [46]. HCV E2 binds to the long extracellular loop of CD81 homodimers, however CD81 homodimers may not be the sole mediator of HCV internalisation and may function to concentrate viral particles on the surface of membranes [47]. The introduction of the HCV pseudoparticle (HCVpp) *in vitro* culture

system led to the identification of SRB1 as another receptor involved in HCV cell entry. SRB1 is a high density lipoprotein (HDL) receptor with a high affinity for HCV E2. Antibodies against SRB1 have been shown *in vitro* to block the interaction between E2 and SRB1 and subsequently prevent internalisation of the HCVpp particle [48]. CLN1 was identified as another HCV co-receptor through screening of CD81+ SRB1+ 293T cells for additional receptors required to make these cells permissive to HCVpp infection [49]. The claudin family of proteins are small membrane-spanning proteins involved in the formation of tight junctions and CLN1 is highly expressed in the liver, where it plays an important role in the formation of paracellular sealing between hepatocytes [50]. siRNA knock-down of CLN1 results in decreased infectivity of HCVpp, further demonstrating its role during HCV cell entry [51].

Another tight junction protein identified as a receptor for HCV is occludin. Although overexpression of occludin in HCVpp permissive cell lines did not render the cells more susceptible to infection, silencing of its expression abolished the ability of the virus to enter the cells [52]. HCV can also bind to GAGs on the cell surface, specifically to GAG heparin sulphate via the E2 protein [53]. Four viral epitopes in the hypervariable region of E2 have been shown to be important for this interaction and treatment of the HCVpp infection model with heparin sulphate could inhibit binding of E2 to GAG heparin sulphate, thus inhibiting viral entry [54]. Another receptor proposed to be involved in viral entry is the LDLR. LDLR primarily binds to cholesterol in the form of LDL circulating in the plasma; ligand binding leads to the internalisation of the receptor-ligand complex via clathrin-mediated endocytosis, with LDL dissociating from the receptor in the low pH environment of the endosome [55]. HCV was shown to bind strongly to LDLR, and antibodies targeting this receptor could significantly inhibit absorption of HCV into the cell [56]. Indeed, single nucleotide polymorphisms (SNPs) in the LDLR have been linked to liver disease severity and response to therapy during treatment of HCV [57].

Upon receptor binding, HCV enters the cell via clathrin-mediated endocytosis and fusion of viral and endosomal membranes in a pH-dependent manner, allowing for viral uncoating and introduction of the viral genome into the cytoplasm of the cell [11]. The viral RNA then undergoes replication or protein translation [58]. Replication of the virus occurs on the ER and as HCV has a positive sense RNA genome, the viral RNA

acts as an mRNA. Replication of the viral genome occurs when the NS5B RdRp synthesises the negative strand of the genome, which then serves as a template for the synthesis of a positive strand of the genome. The positive strand RNA genome is encapsulated and enveloped with viral structural proteins on the ER. Cap-independent translation is initiated upon binding of the 40S ribosomal subunit to the IRES within the 5'NCR. Polypeptide processing of the structural proteins is mediated by ER signal peptidases and NS proteins via the viral proteases NS2-3 and NS3-4A [59,60]. Replication of HCV on the ER causes conformational changes in the ER membrane, leading to the formation of a replication complex, termed the membranous web, consisting of HCV RNA, proteins and altered ER membranes [61].

1.1.4 HCV Genotypes and quasispecies

There are seven genotypes of HCV, which can have as much as 33% divergence in nucleotide sequence [62]. Proteins such as the envelope E1 and E2 glycoproteins exhibit the highest sequence diversity among genotypes, reflecting the demand on these proteins to continually evolve due to pressure from the immune system. Proteins not exposed to the immune system, such as the NS proteins, show less sequence diversity between genotypes [8]. The preferred method of discriminating viral genotype is based on the 5'NCR due to its highly conserved nature, which allows for accurate discrimination between genotypes. However, this conservation means that while tests can distinguish between viral genotypes, genotype subtypes are not readily identified [63].

HCV has a high replication rate and a person chronically infected with HCV is thought to produce up to a trillion new viral particles per day [64]. This high rate of viral production, alongside the high error rate of the RdRp NS5B during replication, leads to the generation of many HCV mutants, which therefore exist together with the original HCV virus sequence [8]. This has led to the generation of many different subtypes within each viral genotype and within an infected individual, the existence of closely related yet genetically distinct quasispecies. Therefore a patient will be infected

with a population of different HCV genomes that are closely related, allowing the virus to effectively evade immune responses [65].

HCV genotype is an important determinant of response to IFN- α therapy, with genotype 1 infected patients having much poorer response rates than patients infected with other viral genotypes [2]. Within genotype 1 infected patients, there are two dominant subtypes: 1a and 1b. Those infected with genotype 1b have a better response to IFN- α therapy than those infected with 1a [66]. Differences between these two subtypes has also been observed in clinical trials for new, direct acting anti-viral drugs [67]. Results from phase III clinical trials with the protease inhibitor telaprevir indicate that those infected with genotype 1b had higher response rates than those infected with genotype 1a [68]. These differences between the subtypes of genotype 1 will have important consequences when treating HCV infected patients in Ireland. It is estimated that 55% of the Irish population are infected with HCV are infected with genotype 1. Although the precise breakdown of the subtypes of those infected with genotype 1 is not documented, it is known that 11.2% (390/3493) represent women who were infected with genotype 1b in the 1970s through contaminated anti-D [69, 71].

1.1.5 History of HCV infection in Ireland

Following the identification of HCV in 1989, a national screening programme was set up to identify individuals who had possibly been infected through contaminated blood products in the previous two decades. This investigation resulted in the positive identification of over 10,000 people infected with HCV, as summarised in figure 1.6. Based on those known to be infected through contaminated blood products, and intravenous drug abusers (IVDA) who undergo testing when they first present at drug treatment clinics, it is estimated that between 20,000 and 50,000 individuals in the Irish population are currently infected with HCV, approximately 0.5–1.2% of the population, a statistic similar to other countries in Northern Europe [69].

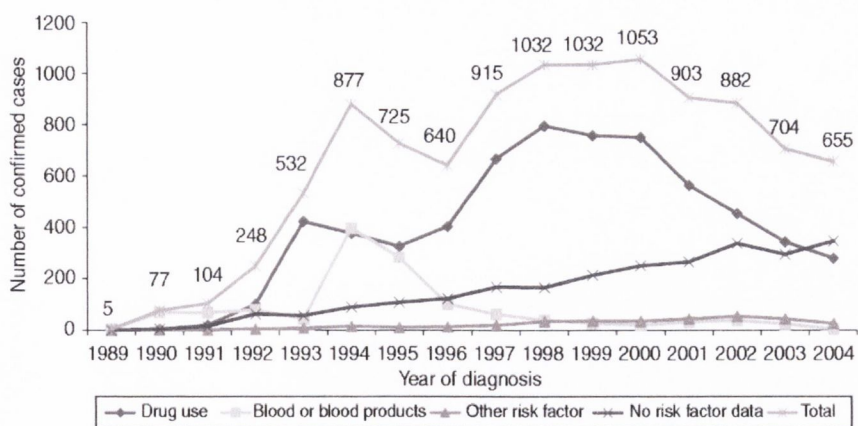


Figure 1.6. Cases of HCV confirmed in Ireland by the National Virus Reference Lab between 1989-2004

Number of cases of confirmed HCV infection in drug users, patients who received contaminated blood products and other patients along with total confirmed cases, diagnosed between 1989 and 2004. Taken from [69].

Over 10,000 cases of HCV were identified in Ireland between the virus being discovered in 1989 up to 2004. Identification of those infected through contaminated blood products peaked in 1994 following a mass screening campaign. Since 1994, the cases in Ireland positively identified for HCV are predominantly within the IVDA cohort. There was also a rise in individuals testing positive who have no known classical risk factors such as IVDA or receipt of a blood transfusion.

The vast majority of newly identified cases in this country are from needle sharing during IVDA, with many of these also co-infected with human immunodeficiency virus (HIV). Co-infection with HIV is a compounding risk factor for development of accelerated symptoms typically associated with long term chronic HCV infection, including cirrhosis and hepatocellular carcinoma. IVDA cases have a lower rate of spontaneous viral clearance during acute infection, due to compromised immunity from HIV co-infection and drug and alcohol abuse [70].

Prior to routine screening of blood products for HCV, HCV infected blood products were administered to people with blood clotting disorders, recipients of blood transfusions and women infected with the virus after receiving contaminated anti-D

immunoglobulin. Irish women infected through contaminated anti-D divide into two cohorts: those infected between 1977 and 1979 and those infected between 1991 and 1994, with the former cohort infected with genotype 1b and the latter cohort infected with genotype 3 of the virus. From the 1970s cohort, 704 women were identified through a mass screening programme to be seropositive for HCV in 1994, with 390 (55%) PCR +ve cases detected for the virus [71]. This represented a high rate of spontaneous clearance during acute infection, possibly due to the fact that those infected were young, healthy women (the average age of infection was 28), who received a relatively low dose of HCV. The number of patients testing PCR +ve from the 1990s cohort is much lower, most likely reflecting the low level of HCV RNA present in the contaminated anti-D batch [72].

Study of the cohort of women infected with HCV genotype 1 through infected anti-D provides an interesting insight into the role of the human immune system in clearance of HCV. What makes these women a unique and homogenous group to study is that they were all infected with the exact same genotype of virus, were all of similar age and health status, and were all of the same ethnicity. Of the 704 women infected with anti-D and tested antibody positive, 314 women developed an adaptive immune response that successfully eliminated the virus, whereas 390 developed an adaptive response but failed to clear HCV [71]. Interestingly, there is a large cohort of women (~55%) who are thought to have been exposed to the virus but are not infected and have no evidence of antibodies against HCV. This indicates that the innate immune system in these women successfully eradicated the virus.

1.2 Anti-viral immunity

HCV has evolved mechanisms to allow it to evade anti-viral immune responses, having major implications for both the natural clearance of the virus and response to IFN- α treatment. The human immune system comprises many effector systems, cells and molecules, spread throughout all organs and systems of the body which defend and protect against infection and disease. This remarkable and complex system is capable of distinguishing between harmful and innocuous, between self and non-self and between

commensal and pathogen. Two highly linked branches, the innate and adaptive immune systems, interact to provide effective anti-viral immunity. When a pathogen initially enters the body, it encounters the innate immune system; this system is a broad range, non-specific first line of defence, capable of successfully eliminating many pathogenic challenges. If the threat persists, the innate immune system activates the adaptive immune system, a more exact defence that can eliminate specific pathogens.

Preventing pathogen entry is one of the simplest means of immune defence. Anatomical barriers include the skin and the mucosal epithelia that line the respiratory and reproductive tracts, which have both physical and chemical means of preventing pathogen entry. Should these defences be breached, underlying epithelial surfaces contain resident macrophages, such as Kupffer cells in the liver, and circulating neutrophils that can rapidly recognise and phagocytose pathogens. Not only can they destroy the pathogen, they are potent producers of cytokines and chemokines, thus invoking an inflammatory response. During inflammation, many processes are activated to limit pathogenic spread, including the coagulation system, endothelial activation and complement system activation. However, should these immediate first lines of defence fail, early innate immune activation is capable of inducing a more potent response through various effector leukocytes and detection of pathogens via pattern recognition receptors (PRRs).

Adaptive immunity differs from innate immunity, as rather than taking hours to activate, it can take days; it is highly specific in the recognition of a pathogen and it is capable of retaining immunological memory. However, effective adaptive immune responses are only elicited following innate immune activation. The effector cells of the adaptive immune system are T and B lymphocytes, each type capable of generating receptors specific for a particular antigen. B lymphocytes recognise antigen via immunoglobulins on their cell surface. When these are expressed on the B cell surface, they are known as the B cell receptor (BCR). Following binding of an antigen to the BCR, the B cell becomes terminally differentiated into a plasma cell and these receptors are secreted and classed as antibodies.

T cells recognise specific antigen epitopes via the T cell receptor (TCR). The TCR is expressed together with a co-receptor, either CD4 or CD8. CD8⁺ T cells are

cytotoxic T lymphocytes (CTLs), which recognise peptides presented by MHC class I molecules and have potent anti-viral activity. CD4⁺ T cells are further differentiated into two classes; Th1 and Th2 cells. Th1 cells control intracellular bacterial infection and promote antibody secretion by B cells. Th2 cells are important for controlling helminth infection and also activate B cells to produce antibodies. The genes for the BCR and TCR are encoded on multiple gene segments and the generation of these receptors require gene rearrangement, enabling them to create receptors of unique specificity. Once a BCR or TCR engages with its specific antigen, the lymphocyte bearing that receptor clonally expands. Some of these clones will persist perpetually in the body, facilitating rapid expansion should the pathogen be encountered again. However many viruses, such as HCV, are constantly evolving and replicate inaccurately, allowing them to constantly evade specific memory T and B lymphocytes, thus making them difficult targets for vaccination.

HCV induces both T and B cell responses and the extent of induction of their activation can determine whether an infected individual becomes chronically infected with the virus. For example, chronic infection is associated with low frequencies of circulating CTLs, whereas individuals who spontaneously clear the virus demonstrate potent and sustained CD8⁺ T cell responses [73,74]. A ‘stunned’ CD8⁺ T cell phenotype has been described in HCV, where virus specific CD8⁺ T cells are activated yet dysfunctional and therefore fail to secrete anti-viral cytokines such as IFN- γ [73]. CD4⁺ T cells also play an important role in the control and resolution of HCV. Chimpanzees that previously resolved HCV infection, failed to clear the virus upon re-infection when Th1 responses were ablated using CD4⁺ T cell specific antibodies [75]. In humans, HCV specific CD4⁺ T cell responses correlate with viral load and disease progression [76]. Furthermore, HCV has been shown to inhibit the secretion of IL-2 by activated T cells, thus preventing IL-2-mediated T cell growth and expansion and differentiation of antigen specific memory T cells [77]. Functional humoral responses are also crucial for HCV viral clearance and have been the subject of intense scrutiny in efforts to develop a successful vaccine. Indeed, rapid induction of neutralising antibodies against HCV envelope proteins is associated with successful viral clearance during acute infection [21,22].

1.3 The anti-viral innate immune system

1.3.1 Innate immune cells involved in anti-viral immunity

Most cells involved in innate immunity are derived from a common myeloid progenitor in the bone marrow which can differentiate into granulocytes; such as neutrophils, eosinophils and basophils; and monocytes, which in turn can differentiate into macrophages and DCs. Innate immune cells derived from common lymphoid progenitors in the bone marrow include natural killer (NK) cells and lymphocytes. Macrophages and neutrophils are efficient phagocytes that recognise, engulf and destroy invading pathogens. Ingestion of pathogens by macrophages upregulates the expression of MHC class II molecules on their cell surface and allow them to become antigen presenting cells (APCs) and present pathogen-derived peptides to CD4⁺ T cells. Therefore, successful activation of adaptive immune responses is dependent on effective detection and presentation of pathogens to the adaptive immune system by cells within the innate immune system. DCs are professional APCs that reside in tissues and peripheral lymphoid organs and are key mediators of innate anti-viral immunity. There are two major types of DCs: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs recognise a wide variety of pathogens and are mainly involved in antigen presentation and naive T cell priming, whereas pDCs respond primarily to viral infection by producing large amounts of potent anti-viral type I IFNs and have been implicated in controlling HCV infection [78].

NK cells are innate immune cells derived from a common lymphoid progenitor with potent anti-viral potential. Invariant receptors on the NK cell surface recognise transformed and virally infected cells and kill targets by releasing the contents of the cytotoxic granules found in their cytoplasm. NK cells are activated in response to type I IFNs and cytokines derived from macrophages, including IL-12, produced early in the activated innate response. IL-12, along with the pro-inflammatory cytokine TNF- α , stimulate NK cells to produce large quantities of the type II IFN, IFN- γ . IFN- γ is a cytokine that exerts anti-viral actions through various processes such as MHC upregulation and Th1 T lymphocyte differentiation. Patients who resolve acute HCV are known to have NK cells with a more activated phenotype than those who do not resolve

infection [79,80]. NKT cells are similar to NK cells, but express an invariant TCR that recognises presentation of lipids via CD1d molecules. Their numbers are few in circulation; however they compose approximately 12% of intrahepatic lymphocytes. Although the function of NKT cells has not been fully elucidated, it has been shown during HCV infection, intrahepatic NKT cell numbers are reduced, as are total NK cell numbers, indicating they are targeted by the virus to inhibit their role in anti-viral immunity within the liver [81,82].

1.3.2 Pattern Recognition Receptors (PRRs)

Although the innate immune response lacks the range of specificities found in adaptive immunity, it can identify certain patterns conserved on pathogens, termed pattern associated molecular patterns (PAMPs), through germ-line encoded receptors PRRs. PRRs are capable of recognising and mounting a quick response to a broad range of pathogens and can be classed into a number of functionally distinct classes including, toll-like receptors (TLRs), nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG)-like receptors (RLRs). In humans, there are ten TLRs; membrane bound receptors that sense a diverse range of PAMPs from bacteria, viruses, fungi and parasites. An interaction between a PAMP with its cognate TLR results in the initiation of a signalling cascade, which can be defined as either dependent or independent of the adapter molecule myeloid differentiation primary response gene 88 (MyD88). Activation of these pathways activates the transcription factors NF- κ B and IFN-regulatory factors (IRFs) [83]. TLRs signal through five toll/IL-1 receptor (TIR) domain containing adapter molecules: MyD88, MyD88-adapter-like (MAL), TIR-domain-containing adapter protein inducing IFN- β (TRIF), TRIF-related adapter molecule (TRAM) and sterile α and armadillo-motif-containing protein (SARM), with SARM being a negative regulator of signalling [84] (Fig.1.7). TLR engagement with its cognate ligand activates MyD88 via the adapter MAL, leading to signalling through IL-1 receptor-associated kinases (IRAKs) and TNF receptor associated factors (TRAFs), which in turn activate NF- κ B. MyD88 independent signalling occurs when the adapter TRAM activates TRIF, which leads to IRF3 and IRF7 activation via the TANK-binding kinase 1/I κ B kinase ϵ (TBK1/IKK ϵ)

complex [85]. Generally, activation of NF-κB is associated with the upregulation of pro-inflammatory genes, whereas the activation of IRFs is associated with the upregulation of type I IFNs.

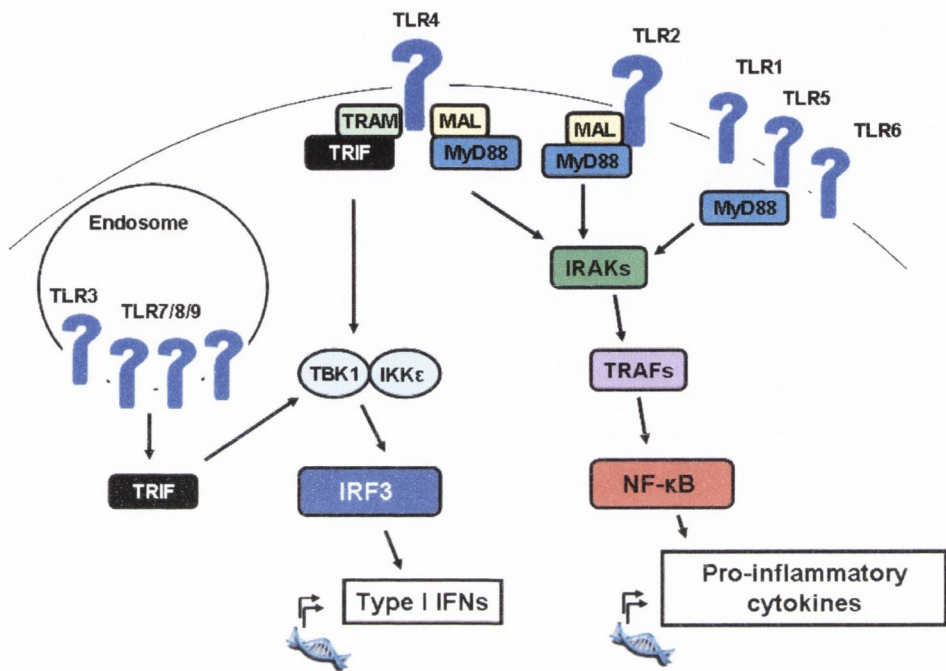


Figure 1.7. TLR receptor activation upregulates type I IFNs and pro-inflammatory cytokines

TLRs found on the cell membrane include TLR1, TLR2, TLR4, TLR5 and TLR6, whereas TLR3, TLR7, TLR8 and TLR9 are found on endosomal membranes. All cell membrane TLRs can signal through the adapter molecule MyD88 to activate IRAK and TRAF family members, which subsequently activate the transcription factor NF-κB, promoting pro-inflammatory gene induction. Endosomal TLRs and TLR4 can signal independent of MyD88 via the adapter molecule TRIF. TRIF activates the TBK1/IKKε complex, which phosphorylates the transcription factor IRF3, allowing them to dimerise and translocate to the nucleus, promoting upregulation of type I IFNs.

RLRs are cytosolic receptors which include RIG-1, melanoma differentiation factor-5 (MDA-5) and laboratory and of genetics and physiology-2 (LGP-2). These receptors signal via the adapter molecule IFN promoter stimulator-1 (IPS-1) (also termed MAVS/VISA/Cardif), which is mitochondrial associated (Fig. 1.8). IPS-1 activates the TBK1/IKKε complex, which in turn activates the downstream transcription factors NF-κB, IRF3 and IRF7 [86].

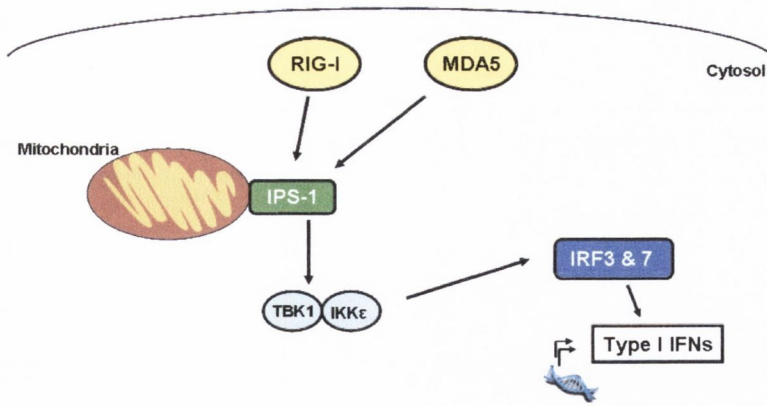


Figure 1.8. Cytosolic RLRs activation promotes type I IFN production

RIG-I and MDA-5 detect cytosolic RNA during viral infection. Activation of these receptors initiates a signalling cascade via the mitochondrial associated IPS-1 and the TBK1/IKK ϵ complex, leading to the phosphorylation and dimerisation of IRF3 and IRF7. These transcription factors subsequently translocate to the nucleus where they upregulate the expression of type I IFNs.

1.3.3 Viral recognition by the innate immune system

Many PRRs in the innate immune system detect and respond to viral PAMPs. These include members from the TLR, NLR and RLR families, as well as some novel cytosolic DNA sensors. Many of these sensors are located on the cell membrane (e.g. TLR2 and 4), while others are found on membranes within endosomal compartments (TLR 3, 7, 8 and 9), while others are located in the cytosol (RLRs and NLRs), allowing the innate immune system to engage with viruses at various stages of their life cycle.

TLR2 and TLR4 are present on the cell membrane and are traditionally associated with response to fungal and bacterial PAMPs. However, a role for these TLRs in viral detection and response has emerged. TLR2 activation has recently been shown to result in the production of type I IFNs, as well as activate pro-inflammatory cytokine production, in response to several DNA viruses [87]. TLR2 also upregulates an inflammatory response upon stimulation with either HCV core protein or NS3 protein [88], however this is not believed to be particularly relevant *in vivo*, as infectious particles failed to elicit the same response [17]. The detection of viral PAMPs by the

endosomal TLRs, TLR3, TLR7, TLR8 and TLR9, is well-established. TLR3 recognises dsRNA, TLR7/8 recognise ssRNA and TLR9 recognises DNA with CpG motifs [89]. TLR3 signals through TRIF upon ligand interaction, whereas the other TLRs signal via MyD88. TRIF activates the transcription factor IRF-3 which, once phosphorylated, dimerises and translocates to the nucleus to upregulate the production of type I IFNs. TLR7 and TLR9 are constitutively expressed within pDCs, an innate immune cell type known for its abundant production of IFN- α upon viral infection [90].

Cytoplasmic RLRs also detect viral nucleic acids. RIG-I, recognises 5' triphosphates on uncapped viral RNA, as opposed to endogenous host mRNA which contains a nucleic modification at the 5' end of its mRNA (a 5'cap), providing mRNA stability. RLRs can recognise both positive and negative sense RNA viruses, including HCV. RIG-I and MDA-5 can recognise dsRNA, depending on the length of the sequence [91]. Viral activation of RLRs results in signalling via the mitochondrial adapter protein IPS-I, which activates IRF-3 and -7, leading to the upregulation of type I IFNs [86]. Viral DNA can also be detected indirectly via RLR, as cytoplasmic RNA polymerase III converts viral DNA to RNA, which consequently activates RLRs [89].

Although cytoplasmic sensors of RNA have been known about for some time, cytoplasmic DNA sensors are only recently being identified. The first to be confirmed was DNA-dependent activator of IFN-regulatory factors (DAI, also called DLM-1/ZBP1) in 2007 by Takaoka et al.; which was shown to bind to dsDNA in the cytoplasm, activate IRFs and promote the induction of type I IFNs [92]. However, this sensor was later shown to be cell type dependent, as knock-down of DAI in certain cell types had no effect on IFN- β induction [93]. Another cytosolic sensor that recognises DNA is Absent in Melanoma 2 (AIM2), a member of the Pyrin and HIN domain-containing protein (PYHIN) family [93,94]. Activated AIM2 forms an inflammasome protein complex that processes the pro-inflammatory cytokine IL-1 β and also induces cell death via caspase 1. Recently described IFI16 is another member of the PYHIN family, but unlike AIM2, IFI16 activates the induction of IFN- β in response to DNA viruses [95]. It is anticipated that other members of the PYHIN family of DNA sensors remain to be discovered, as well as other families of cytoplasmic DNA sensors, which will be important in the detection of viruses by the innate immune system.

1.4 Interferons

IFNs were first identified in the 1950s after it was found that interaction of inactivated virus with cells led to the production a substance that protects against viral infection [96]. Type I IFNs include IFN- α , IFN- β , IFN- ϵ , IFN- κ , IFN- ω , IFN- δ , and IFN- τ [97]. Detection of viruses by PRRs of the innate immune system leads to the rapid production of the type I IFNs, which in turn can upregulate more than 500 genes [3]. Type I IFNs are the largest family of IFNs, with 13 different subtypes of IFN- α and one IFN- β protein in humans; type II IFNs only include IFN- γ ; and type III IFNs have three family members: IFN- λ 1 (IL-29), IFN- λ 2 (IL-28A) and IFN- λ 3 (IL-28B) (Overview of IFNs in Table 1.1). Of the 13 IFN- α subtypes, each subtype appears to have slightly different effects on cell growth and anti-viral activity [98]. All type I and III IFNs can be expressed by most cell types, whereas type II IFN production is limited to activated T cells, macrophages and NK cells [98].

	Subtypes of IFNs	Receptors	Amino acid residues	Expression pattern
Type I IFNs	IFN- α	IFNAR-1/IFNAR2	165–166	Ubiquitously expressed
	IFN- β	IFNAR-1/IFNAR2	166	Ubiquitously expressed
	IFN- δ *	IFNAR-1/IFNAR2	170	Trophoblasts
	IFN- ϵ	IFNAR-1/IFNAR2	208	Uterus, ovary
	IFN- ω	IFNAR-1/IFNAR2	172	Leukocytes
	IFN- κ	IFNAR-1/IFNAR2	180	Epidermal keratinocytes
	IFN- τ **	IFNAR-1/IFNAR2	191	Trophoblasts
	IFN- ζ ***	IFNAR-1/IFNAR2	1618	Spleen, thymus, lymph node
Type II IFNs	IFN- γ ****	IFNGR-1/IFNGR2	146	Activated T cell, macrophage, NK cell
Type III IFNs	IFN- λ 1 (IL-29)	IL-28R α /IL-10R2	200	Ubiquitously expressed
	IFN- λ 2 (IL-28A)	IL-28R α /IL-10R2	200	Ubiquitously expressed
	IFN- λ 3 (IL-28B)	IL-28R α /IL-10R2	196	Ubiquitously expressed

* Only expressed in pigs and cattle
** Expressed in ruminants only
*** Expressed in mice only
**** Acts as a homodimer

Table 1.1. Members of the IFN family

Type I, II and III IFN family members, with their cognate receptors, amino acid length (in humans unless otherwise stated) and expression pattern. Table adapted from [97].

1.4.1 Type I IFNs effector functions

Type I IFNs promote the establishment of an anti-viral state within the immune system. This is done directly through upregulation of intracellular anti-viral proteins and secreted cytokines and chemokines, which promote anti-viral responses in surrounding cells. 100s of ISGs are upregulated by type I IFNs and these genes have a broad range of functions, including direct anti-viral effects, innate immune signalling, metabolism and apoptosis. A summary of the categories of ISGs upregulated is shown in Table 1.2, adapted from [99]. IFN- α and IFN- β upregulate different ISGs, despite signalling through the same receptor [3]. The diverse range of effects exhibited by type I IFNs has led to them being used to treat many different diseases, such as IFN- β treatment for multiple sclerosis and IFN- α treatment for cancer and HCV infection [100].

1.4.2 Type I IFNs and the adaptive immune response

Type I IFNs are critical initiators of the adaptive immune response, and should their function be compromised, leads to defective activation of adaptive responses. T and B lymphocyte maturation and expansion are dependent on the efficient presentation of pathogen epitopes via MHC molecules on APCs. Type I IFNs upregulate the expression of MHC on APCs, along with essential co-stimulatory molecules, and promote the maturation of APCs such as DCs. Anti-viral CD8⁺ T cell activation and proliferation requires the chemokines C-X-C motif chemokine 10 (CXCL10) and CXCR3, which are both strongly upregulated by type I IFNs [101]. Type I IFNs are thought to be essential controllers of T cell responses via their pro-apoptotic properties [102] and are also required for the maturation of B lymphocytes, as they upregulate B cell survival factors and isotype switching following activation [103,104]. Therefore viruses, such as HCV, that perturb type I IFN responses, consequently disrupt the promotion of an effective adaptive immune response.

1.4.3 Type I IFN receptor

How a cell responds to IFN depends on the expression of its cognate receptor on the cell surface. Type I IFNs signal through the IFN- α receptor (IFNAR), a heterodimer that consists of two chains, IFNAR1 and IFNAR2, and activates the JAK-STAT pathway. The IFNAR is ubiquitously expressed. Only one isomer of IFNAR1 exists, however IFNAR2 is known to be alternatively spliced to produce 3 different isomers: a long transmembrane IFNAR2c chain and two truncated forms, a transmembrane IFNAR2b chain and a soluble sIFNAR2a [105]. *In vitro* observations suggest that the IFNAR2b may be a dominant negative regulator of IFNAR2c and that soluble sIFNAR2a may potentiate IFN- α signalling [106]. HCV infection often causes the upregulation of IFNAR1 and IFNAR2 on circulating immune cells [107] and hepatic expression of the IFNAR, determined from liver biopsies of infected patients, is associated with a positive response to IFN- α therapy [108].

Function	number	Function	number
Amino acid metabolism	7	Lipid metabolism	10
Angiogenesis	8	Lymphocyte adhesion	4
Antigen presentation	6	Metabolic enzyme	16
Antigen processing	8	Muscle contraction	4
Anti-viral	8	Nucleotide metabolism	9
Apoptosis	19	Oncogene	6
Blood clotting	5	Protease	7
Cancer	6	Protease inhibitor	6
Cell-cell adhesion	6	Proteasome subunit	6
Cell cycle	7	Protective	8
Chemokine	6	Protein folding	4
Complement	5	Receptor	16
Cytoskeleton	16	RNA splicing	4
Development	18	RNA binding	5
DNA replication/repair	8	Signalling	53
Extracellular matrix	8	Transcription factor	37
G-protein signalling	8	Transcriptional activator	30
Growth factor	15	Transcriptional repressor	8
GTP-binding	7	Translation	11
Hormone	11	Tumour suppressor	4
Host defence	51	Ubiquitination	5
ISG-unknown	10	Unknown	12
Immune modulation	39	Vesicle transport	5
Inflammation	16	Others	22
Ion transport	5	ESTs	78

Table 1.2. Type I IFNs upregulate 100s of genes with diverse functions
 Functional characterisation of the genes upregulated by type I IFNs. The number of genes upregulated in each group is indicated. Data is generated from several microarrays, as described in [99].

1.5 The JAK-STAT pathway

Type I IFNs exert their pleiotropic effects via activation of the JAK-STAT pathway (Fig. 1.9). This signalling cascade is used by many cytokines and growth factors, where receptor associated JAK kinases activate STAT transcription factors, which translocate to the nucleus and bind to specific promoter elements. In mammals, there are four JAK proteins (JAK1–3 and tyrosine kinase 2 [Tyk2]) and seven STAT proteins (STAT1–4, 5a, 5b and 6). JAKs are tyrosine kinases that non-covalently bind to a receptor and become activated upon receptor-ligand interaction. Ligand binding causes receptor dimerisation and this receptor clustering allows associated JAK proteins to auto-phosphorylate. The activated JAK proteins in turn phosphorylate the receptor, creating docking sites for cytoplasmic STAT proteins which bind to these phosphotyrosine residues through their SH2 domains. Receptor bound STAT proteins are subsequently tyrosine phosphorylated by activated JAKs, thus changing their conformational shape, promoting their dimerisation with other STAT proteins in the form of either homo- or hetero-dimers. STAT dimers are imported into the nucleus via their nuclear import signal sequences, where they can bind to particular promoter elements and activate transcription of target genes. This signalling pathway is tightly regulated by three families of negative regulators, protein tyrosine phosphatases, the protein inhibitors of STATs (PIAS) and suppressor of cytokine signalling (SOCS) proteins [109].

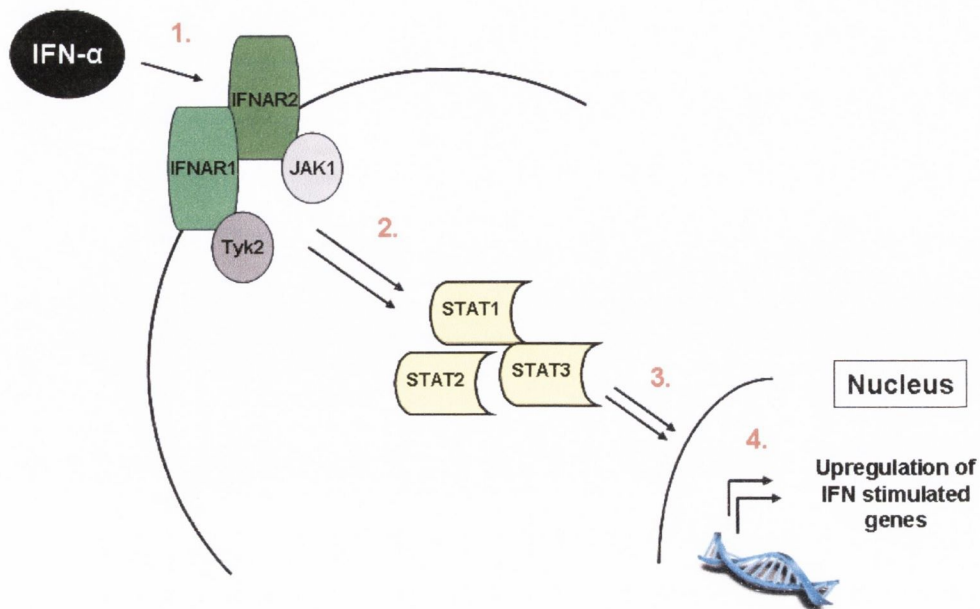


Figure 1.9. IFN-α signals through the JAK-STAT pathway

1. IFN-α binds to the IFN-α receptors (IFNAR1 and IFNAR2), promoting their dimerisation. Receptor clustering allows associated JAK proteins, Tyk2 and JAK1, to auto-phosphorylate, thus becoming activated. Activated JAK proteins phosphorylate the IFNARs, creating docking sites for cytoplasmic STAT proteins. 2. STAT1, STAT2 and STAT3 are activated in response to IFN-α. Binding of STATs to the receptor promotes Tyk2 and JAK1 phosphorylate of receptor-bound STAT proteins. Phosphorylated STAT proteins form homo- or hetero-dimers. 3. Following dimerisation, activated STAT proteins are imported into the nucleus where they bind to specific promoter elements. 4. Binding of STAT dimers to gene promoters leads to the upregulation of over 500 IFN-α stimulated genes within the cell.

1.5.1 JAK proteins

All JAKs contain seven ‘JAK homology’ domains (JH1-JH7) and are ubiquitously expressed with the exception of JAK3, which is only found in cells derived from myeloid and lymphoid lineages. The JH1 domain possesses tyrosine kinase activity, whereas the JH2 domain is a pseudokinase domain that is thought to regulate the catalytic activity of the JH1 domain. JH3 and C-terminal portion of JH4 form the SH2 domain and the N-terminal portion of JH4 spanning to JH7 domain assemble into a FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domain that is involved in receptor binding (Fig.1.10).

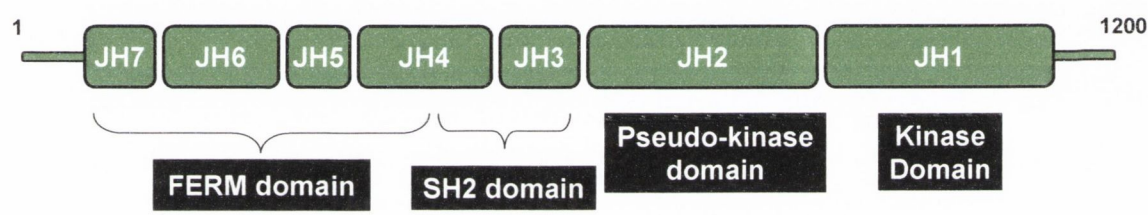


Figure 1.10. Structure of JAK kinases

Each JAK protein contains 7 domains, JH1-JH7. The JH1 domain has kinase activity; the JH2 domain is a pseudokinase domain involved in regulation of the JH1 domain; JH3 and part of JH4 form an SH2 domain; which recognises phosphorylated proteins; and the rest of JH4 spanning to JH7 forms a FERM domain, involved in receptor binding.

In type I IFN signalling, Tyk2 and JAK1 associate with the cytoplasmic portions of IFNAR1 and IFNAR2 (Fig. 1.9). There is some redundancy in this system as Tyk2^{-/-} mice show little difference in resistance to viral infection compared to wild type (WT) [110]. Although these mice had defective responses to type I IFNs, they were not completely abrogated. In contrast, JAK1 is more pleiotropic and JAK1^{-/-} mice display an early post-natal lethal phenotype and JAK1^{-/-} embryonic fibroblasts are completely unresponsive to type I IFN [111]

1.5.2 STAT proteins

The members of the STAT family share 6 domains that are structurally and functionally conserved; these include N-terminal, coiled-coiled, DNA binding, linker, SH2 and a transcriptional activation domain (Fig. 1.11).

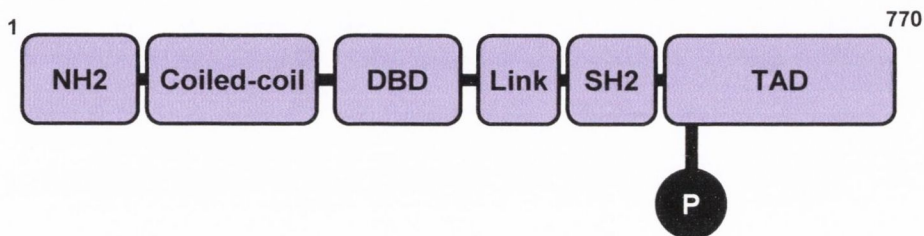


Figure 1.11. Overview of STAT protein structure

STAT proteins contain six functional domains. At the N terminus is the NH2 domain, involved in STAT protein oligomerisation. This is followed by a coiled-coil domain and then a DNA binding domain (DBD), a linker domain (link) and an SH2 domain, which recognises phosphorylated proteins. At the C terminus is the transcriptional activation domain (TAD), which contains the STAT phosphorylation sites.

Inactive STAT proteins form homotypic dimers in the cytoplasm through their N-terminal domains, thus regulating and stabilising STAT proteins in the absence of stimulation [112]. The coiled-coiled domain of STAT proteins interacts with other proteins and has been implicated in the nuclear import of STATs. The DNA binding domain mediates STAT binding to DNA promoter elements while the linker domain links the DNA binding domain to the SH2 domain. The SH2 domain is found in all proteins that bind to phosphorylated tyrosine residues and it is through this domain that STAT proteins bind phosphorylated docking sites on cell membrane receptors, activated JAKs and other STAT proteins, to form homo- or hetero- dimers. Finally, the transcriptional activation domain is found at the carboxyl terminus and contains tyrosine and serine residues that become phosphorylated during signalling [112]. Alternative splicing produces STAT proteins which lack C terminal transcriptional activation domain. These truncated STAT proteins are thought to act as dominant negatives on full-length protein function, however recent *in vivo* studies suggest that

they may be alternatively activated by cytokine signalling to upregulate different subsets of genes compared to their full length forms [113].

STAT knock-out (KO) mice have given insight into the diverse roles played by STAT proteins in many cell processes. STAT1^{-/-} mice exhibit signalling defects in response to type I IFNs which leave the mice susceptible to viral infection [114,115]. STAT2^{-/-} mice are also more susceptible to viral challenge and have defective type I IFN responses [116]. STAT3 deficient mice die early in embryogenesis before gastrulation, but conditional knock-down has revealed its role in many biological functions such as cell growth, suppression of apoptosis and cell motility [117,118]. STAT4 and STAT6 are important for T cell subset polarisation and STAT4^{-/-} and STAT6^{-/-} mice have defective T cell subset distribution. STAT5a deficient mice showed the importance of STAT5a in mammopoietic and lactogenic signalling, whereas STAT5b was shown to be crucial in the sexual dimorphic effects of growth hormones in liver and other tissue [119].

1.5.3 STAT proteins and IFN- α

STAT1 and STAT2 are the most studied STAT proteins in relation to IFN- α signalling. STAT1 and STAT2 form heterodimers upon IFN- α signalling and these heterodimers subsequently bind to the transcription factor IRF9, forming the IFN stimulated gene factor (ISGF)-3. ISGF-3 binds IFN stimulated response elements (ISREs) in the promoter elements of genes, which leads to the upregulation of ISGs [120]. The role of STAT3 in relation to IFN- α has not been as well established, although it is postulated to play an important role in the anti-viral response activated by IFN- α . In a Daudi lymphoblastoid cell line deficient in STAT3, cells were IFN- α resistant despite having normal STAT1 and STAT2 levels. Restoration of STAT3 restored responsiveness to IFN- α [117]. STAT3 overexpression has been shown to promote the formation of STAT1:STAT3 heterodimers in response to IFN- α , preventing STAT1 from forming homodimers. This resulted in a decreased expression of pro-inflammatory genes associated with STAT1 homodimers while anti-viral ISGs upregulation remained unaltered [117]. These results may explain the observation that STAT1^{-/-} mice are unresponsive to IFN; as these mice would lack both STAT1:STAT1 homodimer and

STAT1:STAT3 heterodimer formation [118]. STAT4 and STAT5 can also be activated by the type I IFNs, although this is limited to specific cell types [121,122].

1.5.4 Anti-viral effector proteins: PKR, OAS and MxA

Over 500 genes are upregulated by type I IFNs [3] and three well-characterised ISGs with potent anti-viral activity are PKR, OAS and MxA. PKR is located in the cytoplasm of cells and is activated in response to dsRNA larger than 30bp in length [123]. PKR exerts its anti-viral effects by inhibiting protein synthesis and can induce pro-apoptotic effects. When PKR binds dsRNA and becomes activated, it dimerises, allowing it to subsequently autophosphorylate, catalysing the phosphorylation of eukaryotic initiation factor 2- α (eIF2- α) on serine 51. This blocks translation in two ways: firstly, it reduces the cellular pool of functional eIF2, which is necessary for all cellular protein translation; secondly, it disrupts the critical delivery of methionyl-tRNA to the 40S ribosome which is critical for translation activation [124].

2'5'-OAS activates the latent endoribonuclease RNaseL, leading to translational suppression through cleavage and subsequent inactivation of 28S rRNA. 2'5'-OAS is expressed in a latent, inactivated form in the cytoplasm and is activated in response to dsRNA. 2'5'-OAS polymerises ATP to 2'5'-linked oligomers of adenosine (2-5A). These 2-5A molecules activate RNaseL, which dimerises and mediates RNA degradation [125]. This RNA degradation amplifies the type I IFN response as degraded RNA activates nucleic acid sensors in the cytosol such as RLRs.

MxA is thought to prevent the replication of many RNA viruses, such as influenza. This is postulated to occur in the early stage of viral life cycle soon after viral entry and prior to genome amplification. MxA recognises viral nucleocapsids and renders them redundant by wrapping around the viral structure and forming MxA/nucleocapsid oligomers [126]. Another anti-viral mechanism proposed for MxA is that it may direct nucleocapsids to alternative sites in the cytoplasm, where they are not functional for RNA synthesis and are likely to be immobilised and subsequently degraded [127]. Interestingly, we have demonstrated that the core protein of HCV co-localises with MxA in a granular pattern in the cytoplasm of cells, a phenomenon that is potentiated with the co-treatment of IFN- α and ribavirin, the drugs used to treat HCV

infection [128]. This was specific for MxA, as PKR and OAS were not affected, revealing a potentially specific anti-viral role for MxA in HCV infection.

1.6 Viral immune evasion

Due to constant pathogen exposure, the immune system has evolved to protect against infection and disease. However, invading pathogens concurrently evolved mechanisms to subvert and evade the immune response. Viruses have a particularly rapid replication rate and high mutation frequency which allow many viruses to effectively avoid anti-viral immunity. Viruses can target many stages of anti-viral immunity, from mimicking inhibitory components of the complement cascade to interfering with antigen presentation via MHC class I [129,130]. An important evasion mechanism frequently utilised by many viruses is targeting innate immunity by inhibiting the upregulation and functions of type I IFNs.

1.6.1 Viral inhibition of TLR signalling

Viruses can block the upregulation of type I IFNs by directly inhibiting signalling cascades activated from TLR and RLR ligand engagement. Vaccinia Virus (VACV), a large dsDNA virus predicted to encode for over 260 viral proteins, has many well documented strategies for blocking TLR signalling. One VACV protein, A46, contains a TIR domain which allows it to interact with the TIR domains of the adapter molecules MAL, MyD88, TRIF and TRAM, therefore preventing signal transduction from TLRs, thus blocking upregulation of type I IFNs and pro-inflammatory cytokines [131]. Another protein from VACV virus, A52, inhibits TLR signal transduction by interacting with IRAK2 and TRAF6, two molecules activated by TLRs as part of the signalling cascade [132]. The importance of A46 and A52 to the viral life cycle of VACV is demonstrated by the fact that deletion of either of the genes encoding for these proteins leads to viral attenuation. Proteins from Hepatitis B Virus (HBV) can inhibit TLR3 and TLR4 signalling in various liver cell types via suppression of IRF3 activation [133]. Flaviviruses have also evolved means of blocking TLR signalling. The NS1

protein of West Nile Virus (WNV) inhibits signal transduction following TLR3 activation by blocking the translocation of IRF3 and NF- κ B [134].

HCV proteins target TLR signalling to evade innate immune activation (Fig. 1.12). The serine protease NS3-4A inhibits TLR3 and MyD88-independent signalling by targeting the signalling molecule TRIF, which is cleaved into two polypeptides, thus destroying functionality [135]. This prevents NF- κ B and IRF3 activation and subsequent type I IFN production. Another HCV protein, NS5A, can also diminish TLR signal transduction by binding directly to the adapter molecule MyD88 via its ISDR domain, preventing recruitment of IRAK1 to the molecule, therefore preventing the upregulation of pro-inflammatory cytokines such as IL-6, but not IFN- β which is upregulated in a MyD88-independent manner [136].

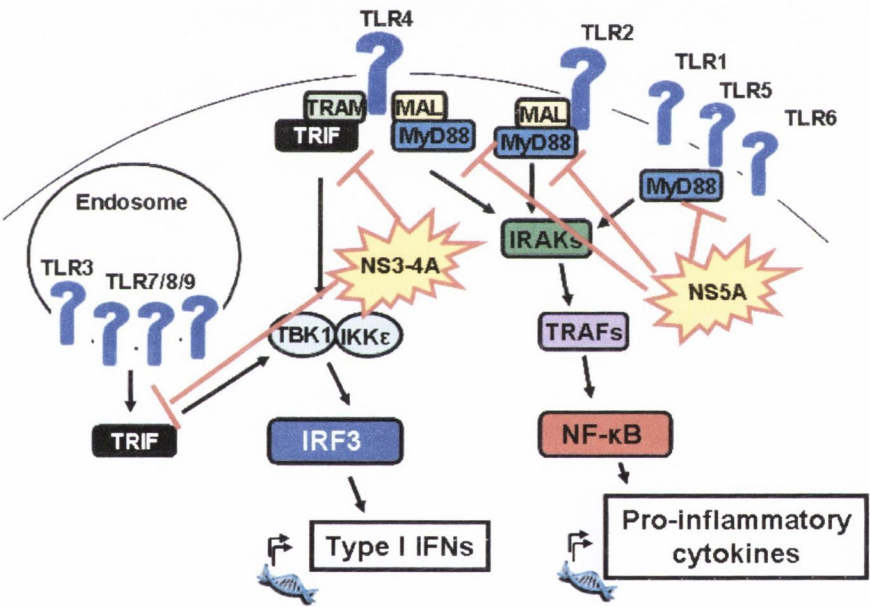


Figure 1.12. HCV NS3-4A and NS5A block TLR signal transduction

HCV NS3-4A protease inhibits upregulation of type I IFNs following TLR activation by targeting the essential adapter molecule TRIF and cleaving it into two polypeptides, thus rendering it inactive. HCV NS5A inhibits MyD88 and subsequently blocks the upregulation of pro-inflammatory cytokines following TLR activation.

1.6.2 Viral inhibition of RLR signalling

Viral targeting of RLR pathways is another effective way of preventing activation of innate immune anti-viral responses. As RLRs are cytoplasmic nucleic acid sensors, they are an important class of detectors of viral infection within a cell. They are also potent activators of type I IFNs. Consequently, many viruses have evolved strategies to counteract RLR activated signalling pathways, including interacting with molecules involved in signal transduction and suppressing synthesis of type I IFNs. One means of preventing recognition of viral RNA by RLRs is by encoding viral proteins that will bind to dsRNA directly, thereby acting as decoys for these receptors without activating anti-viral signalling pathways. The VP35 protein inhibits PKR activation, which is reversible by introduction of deletions and mutations to the dsRNA-binding domain, highlighting the significance of the dsRNA-binding activity of this protein in antagonising innate immune mechanisms. Viruses often target RLRs directly to block their activation. MDA5, an important cellular RLR sensor of Picornoviruses, is targeted by Poliovirus for degradation in a caspase- and proteasome- dependent manner, thus preventing type I IFN production [137]. Furthermore, NS1 protein of Influenza virus prevents activation of RIG-I by targeting Trim 25, an E3 ligase important in controlling RIG-I activation [138].

C6 protein of VACV suppresses upregulation of type I IFNs via RLR signal inhibition. C6 interacts with scaffold proteins that form the TBK1/IKK ϵ complex which, when activated by upstream signals for RLRs and cytosolic DNA sensors, phosphorylate IRF3 and IRF7 [139]. HCV suppresses RLR signalling by targeting the mitochondrial associated IPS-1 molecule, thus blocking type I IFN upregulation. HCV NS3-4A protease specifically cleaves IPS-1 from the mitochondrial membrane thus rendering it unable to activate the downstream TBK1/IKK ϵ complex (Fig. 1.13). HCV is not the only virus encoding a protein that can disrupt IPS-1; Influenza virus suppresses IPS-1 via the PB1-F2 protein and Hepatitis A virus (HAV) cleaves IPS-1 via the protease 3ABC [140,141]

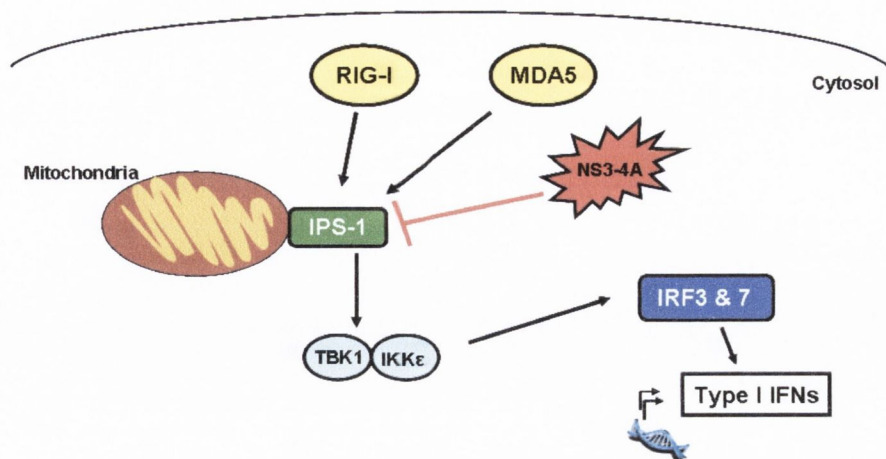


Figure 1.13. HCV NS3-4A inhibits RLR-mediated activation of Type I IFNs

The HCV protease NS3-4A cleaves the mitochondria associated IPS-1 adapter protein, rendering it dysfunctional. This results in diminished ability of RLRs, such as RIG-I and MDA-5, from activating the TBK1/IKK ϵ complex, thus preventing the activation of IRF3 and IRF7 and subsequent type I IFN upregulation.

RLR and many TLR pathways activate IRF3 and IRF7, which are responsible for binding to promoter regions in the IFN- β and IFN- α genes to upregulate their expression. As this is an important convergence of several virally-activated innate immune pathways, many viruses encode proteins that target these transcription factors. Examples include the V protein of Paramyxoviruses, such as Mumps and Parainfluenza, which mimic IRF3 and thus act as a decoy substrate for the TBK1/IKK ϵ complex and prevent the complex from activating IRF3 [142]. Proteins from Herpes simplex virus (HSV) sequester IRF3 by directly binding to it, thereby preventing its activation and promoting its degradation [143]. To date, there is no evidence that HCV can directly interact with IRFs to inhibit type I IFN production.

1.6.3 Viral targeting of IFN signal transduction

Since type I IFNs induce 100s of ISGs through activation of the JAK-STAT signalling cascade, viruses often target this pathway before this comprehensive anti-viral response becomes activated. VACV encodes a soluble IFNAR homologue called B18R, which competes with the receptor to bind type I IFNs [144]. However, most viruses evade the action of IFN- α by targeting intracellular JAK and STAT proteins. Viruses that target JAK proteins include Human Papilloma virus, which targets Tyk2 via an E6 protein, and Human Cytomegalovirus, which interacts with JAK1 to prevent its activation [145].

Viruses that target the JAK-STAT pathway often evolve mechanisms for degrading STAT proteins. Paramyxoviridae is a family of viruses that have done this successfully by targeting STATs for proteasome-mediated degradation via their V protein. Parainfluenza virus 5 V protein preferentially degrades STAT1, whereas type 2 human Parainfluenza virus V protein degrades STAT2 [146]. Respiratory syncytial virus (RSV) also targets STAT2 for proteasomal degradation to inhibit type I IFN signalling [147]. Mumps virus V protein is unique in the Paramyxoviridae family as it can target both STAT1 and STAT3 to completely inhibit IFN- α signalling [148]. HCV has also been shown to target STAT1 to evade anti-viral innate immunity. Phosphorylation of serine 727 on STAT1 was inhibited by the HCV NS3-4A protease in Huh-7 cells [149]. In addition, HCV core protein has been shown to interact with the STAT1 SH2 domain [150]. HCV can also target STAT1 methylation through upregulation of protein phosphatase 2A (PP2A) [151]. HCV-induced upregulation of PP2A resulted in decreased methylation of STAT1, therefore decreasing its ability to bind to DNA and increasing its association with the negative regulator PIAS1. Although *in vitro* HCV expression has been shown to target STAT1 expression in cell lines, it is unknown whether it does so in HCV infected individuals and whether this impacts on IFN- α therapy. Another outstanding issue is that IFN- α activates other STAT proteins as well as STAT1 and the impact of HCV on these proteins remains undefined.

1.6.4 Viral targeting of type I IFN induced effector molecules

Viral inhibition of type I IFN induced effector molecules, such as PKR and OAS, are common mechanisms used by viruses to inhibit the innate immune response. Viruses that target PKR include Adenoviruses, HSV, Influenza A virus and Reoviruses, which often encode more than one protein that counteracts this important viral regulator [152]. HCV NS5A and E2 both target PKR, an astounding fact given that HCV only encodes ten proteins. NS5A interacts through the ISDR and downstream amino acids with the protein kinase catalytic domain of PKR, rendering the anti-viral sensor unable to inhibit protein translation and therefore block viral replication [153]. E2 contains a sequence similar to the phosphorylation site in PKR, thus allowing E2 to bind to PKR and inhibit its activity [154].

Viruses also target OAS-RNaseL to avoid destruction. HSV Type 1 encodes Us11 protein late in its viral life cycle, which directly interacts with OAS, inhibiting the synthesis of 2’-5’ oligoadenylates and therefore preventing the formation of active RNaseL homodimers [155]. HCV NS5A can also bind directly to OAS, although unlike NS5A binding to PKR, it interacts with it through amino acids in the N-terminal of the protein, as illustrated in figure 1.14 [156].

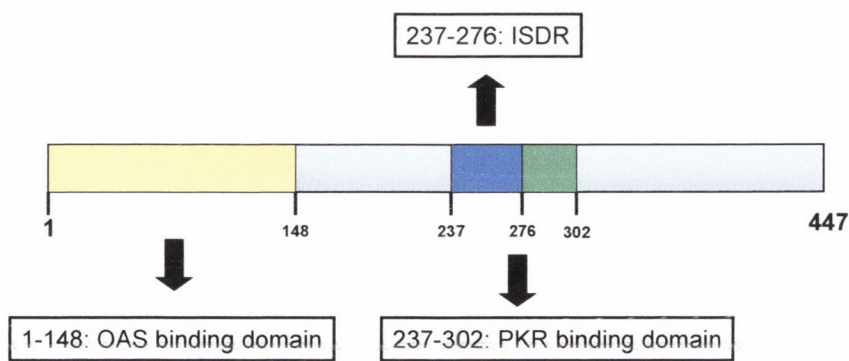


Figure 1.14. Overview of HCV NS5A domains that interact with PKR and OAS

NS5A interacts with OAS through amino acids 1-148, whereas it exhibits its inhibitory activity on PKR through binding via amino acids 237-301, which includes the IFN sensitive determining region (ISDR).

There is little evidence that viral proteins directly interact with and inhibit MxA, possibly due to the fact that this protein has only recently been well described and its full role during viral infection is still being characterised. West Nile virus can shield viral particles from MxA detection by forming cytoplasmic membrane structures, however it does not interact directly with MxA [157]. There has been no documented evidence that HCV directly inhibits MxA, although they are known to co-localise [128].

1.7 HCV therapy: IFN- α

The current licensed therapy for HCV patients is IFN- α in combination with the nucleoside analogue ribavirin. While this therapeutic regime is effective for viral clearance in some patients, particularly those infected with genotypes 2 and 3, it is suboptimal in other patients infected with difficult-to-treat genotypes such as genotype 1 and genotype 4 [2]. The administered IFN- α is covalently attached to polyethylene glycol (PEG) chains, which shields IFN- α from the host immune system, preventing its degradation by metabolic enzymes and prolongs the residence of IFN- α in the body [158]. Ribavirin is thought to be incorporated into the viral genome and increase the mutation frequency during viral replication [159]. Ribavirin has also been found to act as a viral mutagen by broadly introducing errors into the HCV genome, even into highly conserved regions such as the NCRs [128]. Interestingly, ribavirin may also directly affect anti-viral gene expression: in HCV cell culture models, ribavirin both blocked HCV replication and upregulated a distinct set of ISGs in a mechanism independent of IFN signalling, RLRs and TLRs [160]. We have found that ribavirin specifically upregulates the expression of the ISG MxA, which colocalises with HCV in a granular manner, indicating a novel mode of action [128]. The upregulation of MxA by ribavirin was independent of PKR, OAS and CXCL10, the other ISGs examined, suggesting that the upregulation of MxA by ribavirin occurs via an alternative pathway to JAK-STAT that results in the upregulation of a distinct subset of genes.

1.8 Rationale and overall objective

1.8.1 Rationale

IFN- α is a potent activator and mediator of the innate anti-viral response and therefore, should be a highly effective therapeutic for combating HCV infection. Furthermore, the rapid induction of IFN- α upon viral infection would suggest that HCV should be effectively eliminated from the host and not establish chronic infection. The failure of the many infected individuals to naturally clear HCV infection, as well as the 50% of genotype 1 infected patients who fail to respond to therapeutic IFN- α , suggests that HCV has evolved highly effective mechanisms of subverting the action of IFN- α to evade anti-viral immune responses. This must have profound implications for patients receiving IFN- α therapy and viral targeting of IFN- α signalling may be the reason many people fail to respond to IFN- α therapy.

1.8.2 Hypothesis

Our hypothesis is that HCV, in particular viral genotype 1, blocks IFN- α signalling by targeting STAT proteins, specifically STAT3, for degradation, thus inhibiting anti-viral IFN- α signalling. This mechanism would impact on ISG induction and anti-viral innate immunity. We postulate that blocking the specific sites within the virus that mediate STAT3 degradation would recover ISG expression, thus identifying novel therapeutic targets and revealing an important role for STAT3 in anti-viral innate responses. We believe that analysis of ISG induction in immune cells from HCV patients will identify patients who will respond to clinical IFN- α treatment and therefore not need triple therapy with the new protease inhibitors. This would also identify patients who would fail to respond to IFN- α therapy and therefore identify the patients who would need treatment with additional anti-viral drugs such as protease inhibitors.

1.8.3 Specific aims

The specific aims of this project are:

1. To elucidate the molecular mechanism used by HCV to target IFN- α signalling by studying the effect of HCV expression on STAT proteins. This would have serious consequences regarding anti-viral immune responses and therefore both the spontaneous clearance of viral infection and response to IFN- α therapy.
2. To examine if small peptide inhibitors can block HCV-mediated degradation of STAT proteins and potentially recover IFN- α induced anti-viral gene induction.
3. To analyse the expression of IFN- α induced anti-viral genes in immune cells from HCV infected patients who have undergone IFN- α therapy.
4. To determine whether the responsiveness of immune cells to *in vitro* IFN- α stimulation from HCV patients, prior to commencement of clinical IFN- α therapy, is indicative of patients' response to clinical IFN- α therapy.

Chapter 2

Materials and Methods

Chapter 2 Materials & Methods

2.1 Materials

2.1.1 General Materials

General lab chemicals were purchased from Sigma, USA, with the exception of Ficoll-Paque separation medium (Amersham, UK), Phosflow Perm Buffer III and cytofix (BD Biosciences, USA), RNA/DNA free H₂O, NuPAGE® MOPS SDS Running buffer and Pre-cast BIO-RAD Criterion™ 4-12% Bis Tris gels (BioSciences, Dublin), MG132 (Calbiochem, USA), agarose, BSA, EtBr (Fisher, USA), TRIzol®, DH5α competent E.Coli cells, One Shot® Top 10 chemically competent E.Coli cells (Invitrogen, USA), Western blotting molecular weight marker (LI-COR, Germany) PVDF membrane, BCA protein assay reagent (Millipore, USA), Oligo dT, Omniscript RT Kit (Qiagen, Germany), Passive lysis buffer (MSC, Dublin), luciferin (Biosynth, Switzerland), Coelenterazine (VWR international, Dublin) STAT3 scrambled and STAT3 siRNA (Santa Cruz Biotechnologies, USA) SyBr green and IFN-α2a (Roche, Switzerland)

2.2.2 Cell lines/constructs

Huh-7s cells expressing T7 polymerase was a kind gift from Prof. Ralf Bartenschlager, (University of Heidelberg). The HCV plasmid pBRTM/HCV1-3011 DNA construct was a kind gift from Prof. Charles Rice (The Rockefeller University, USA). The empty vectors pBR322 and pcDNA3.1 were both purchased from Invitrogen, USA. pRL-TK was purchased from Promega, USA. ISRE-luciferase and GAS-luciferase were purchased from Stratagene, USA.

2.2.3 Antibodies

For flow cytometric analysis, the PE conjugated antibodies anti-STAT1 pY701 (612564) and anti-STAT3 pY705 (612569) were both purchased from BD Biosciences, USA. For western blotting, anti-STAT1 (9172) and anti-STAT2 (4594) were purchased from Cell Signal, USA, anti-STAT3 (sc 482) was purchased from Santa Cruz, USA, and anti- β -actin (85441) was purchased from Sigma, USA.

2.2.4 Cell culture reagents

DMEM, RPMI, Pen-strep and trypsin-EDTA were all purchased from Biosciences, Dublin. FCS was purchased from Analab, Ireland. Lithium heparin tubes for blood collection were purchased from BD Biosciences, USA. Trypan blue was purchased from Sigma USA, zeocin was purchased from Invivogen, USA and Lipofectamine 2000 was purchased from Invitrogen, USA.

2.2 Patient information

2.2.1 Patient recruitment

Patients attending the HCV out-patient clinics at St. Vincent's University Hospital (SVUH), Dublin and St. James's Hospital (SJH), Dublin were invited to participate in this study. All patients enlisted were HIV negative. Healthy controls were kindly donated from both the staff at SVUH or from volunteers within Trinity College Dublin.

2.2.2 Ethical approval

This study received ethical approval from the local Research and Ethics Committees at SVUH and SJH, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each patient and healthy control before entry into the study.

2.2.3 Treatment Regime

Patients treated with IFN- α therapy received weekly subcutaneous injections of 180 μ g PEG-IFN- α 2a or 1.5 μ g/kg PEG-IFN- α 2b. Genotype 2 and 3 patients receiving PEG-IFN- α 2a were given 800mg ribavirin per day and genotype 1 infected patients received 1000mg for <75kg or 1200mg for >75kg of ribavirin per day. Patients receiving PEG-IFN- α 2b were given 800mg for <65 kg; 1000mg for 65-85 kg, 1200mg for 86kg-105kg, and 1,400mg for >105kg of ribavirin per day.

2.2.4 Antibody and PCR testing

To determine HCV antibody status of patients, all were tested using a third-generation immunoassay (Abbott Diagnostics, Germany) which was confirmed using a third-generation recombinant immunoblot assay (RIBA-3) (Chiron Corp., CA, USA) [161]. PCR status was assessed using the Cobas Amplicor PCR system (Roche Diagnostic Systems, Nutley, NJ), a quantitative real-time PCR that measures HCV nucleic acid in the serums of patients.

2.2.5 Sample Collection

All blood samples were collected in lithium heparin tubes. These tubes were used as heparin activates anti-thrombins, therefore blocking the coagulation cascade and preventing clotting of the blood. 30mls of blood was taken from each subject.

2.3 Methods

I performed all experimental methods and analysis of results described. Design of experiments was done by me in collaboration with my supervisors Prof. O'Farrelly and Dr. Stevenson.

2.3.1 PBMC isolation

PBMCs were isolated from 30ml of whole blood via density gradient centrifugation using Ficoll-Paque PLUS separation medium and Leucosep® separation tubes. Ficoll-Paque PLUS separates mononuclear cells and lymphocytes from erythrocytes and granulocytes. Following centrifugation, erythrocytes and granulocytes cells will sediment at the bottom of the separation medium, whereas the more buoyant PBMCs will be enriched in the interphase above the separation medium. Leucosep® tubes contain a porous polyethylene barrier which prevents the mixture of the sample material with the separation medium, thus decreasing instances of red blood cell contamination.

Ficoll-Paque PLUS, 15mls, was added to a Leucosep® tube and centrifuged at $300 \times g$ for 3 minutes (mins); causing the Ficoll-Paque PLUS to pass through the porous barrier. 15-20mls of blood was added to the tube and centrifuged at $1,580 \times g$ with the brake off for 5mins. Following this, the sedimented erythrocytes and granulocytes are found beneath the porous membrane with the Ficoll-Paque, whereas PBMCs remain above the membrane. PBMCs were harvested with a Pasteur pipette and washed twice in 20ml Roswell Park Memorial Institute (RPMI) solution, with centrifugation at $453 \times g$ for 5mins. Following washing, the cell pellet was resuspended in 1ml of RPMI supplemented with 2% foetal calf serum (FCS). Viable cells were counted using trypan blue on a haemocytometer under a light microscope.

2.3.2 Culture of Huh-7 cell line

For cell line analysis, Huh-7s that stably express the T7 polymerase were used. Huh-7s are a hepatocyte cellular carcinoma cell line derived from a 57 year old Japanese male in 1982 [162]. Huh-7s were cultured in 10% Dulbecco's Modified Eagles Medium (DMEM supplemented with 10% FCS and 250U/ml penicillin, 250µg/ml streptomycin, 6µg/ml zeocin). Cells were maintained in a humidifier at 37°C and supplied with 5% CO₂. Cells were passaged every 2-3 days when reaching ~80% confluence. Upon reaching confluence, cells were removed from their flask by incubating with trypsin-EDTA for 5-10mins at 37°C. The cells were resuspended in 10% DMEM and centrifuged at 290 x g for 5mins. The cell pellet was resuspended in 10% DMEM and viable cells were counted using trypan blue on a haemocytometer under a light microscope before being transferred to a sterile flask.

2.3.3 Stimulations

IFN- α was prepared from Roferon-A (IFN- α -2a). The stock concentration was 6×10^6 IU/ml, which was diluted 1:6,000 to give a final concentration of 1000IU using RPMI for PBMCs or DMEM for Huh-7 cells. For MG132 treatment, cells were treated with 10µM MG132 for 2h. Prior to all treatment, cells were rested in RPMI or DMEM supplemented with 2% FCS for 2h. This serum starvation synchronises the cells into the same phase of cell cycle.

2.3.4 Peptide synthesis and reconstitution

All peptides were synthesised by GenScript Corporation (Piscataway, New Jersey, USA) and had >95% purity. Each peptide was designed to have a nine arginine tail at the N-terminus to facilitate cell entry. Peptides were diluted to a concentration of 2mM in nuclease-free (NF) H₂O, aliquoted and stored at -20°C. For working stock, each aliquot was diluted to a concentration of 0.42mM in 34% dimethylformamide (DMF) in NF-H₂O, due to the hydrophobic nature of the peptides. DMF was used instead of

dimethyl sulfoxide (DMSO) to dissolve the peptides as some of peptides contained cysteine residues, which DMSO could thiol oxidate resulting in possible dimerisation of peptides. Peptides synthesised are represented in Table 2.1.

	Elongin C (p7)	Cul-2 (E2)	Elongin C (NS2)
Peptide	9R-VSFLVFFCFAYL	9R-SWAIKWEYVVLFLLL	9R-VQGLLRIC
Scrambled	9R-WVFLFFFYLACSV	9R-YVLFLSELKWLI AVLW	9R-CVIGLQLR

Table 2.2. List of peptides synthesised

Peptides were designed against the elongin C binding sites in the p7 and NS2 protein and a peptide was designed against the cul-2 binding site in E2. Corresponding scrambled peptides were also designed. Each peptide contained a 9-polyarginine tail to facilitate cell penetration (9R).

2.3.5 Huh-7 cell transfection

For transfection of Huh-7 cells, 6 well plates were seeded at a density of 0.3×10^6 cells per well in DMEM supplemented with 10% FCS only. Cells were transfected 24h later with 0.8 μ g of indicated vector for 16h using Lipofectamine 2000 at a vector:lipofectamine ratio of 1:2. 1.6 μ l of lipofectamine was incubated at room temp with 248 μ l serum-free (SF) DMEM for 5mins. 250 μ l of appropriate vector dilution was added and incubated for a further 20mins before being added slowly to cells.

For 96 well plates, Huh-7 cells were seeded at a density of 1×10^5 cells/well in DMEM supplemented with 10% FCS. 24h later, cells were transfected with 230ng DNA. For each well, 0.4 μ l lipofectamine was added to 9.6 μ l SF-DMEM and incubated for 5mins. 10 μ l of DNA diluted in SF-DMEM was added to the lipofectamine mix and incubated at RT for 20mins before being added to cells.

No antibiotics were used at any stage for cells being transfected.

2.3.6 RNA extraction and quality control

Total RNA was isolated from PBMCs and Huh-7s following the TRIzol® reagent manufacturer's protocol (Invitrogen, CA, USA). Briefly, cells were resuspended in 1ml of TRIzol® reagent and incubated at RT for 5mins. 200µl of chloroform was added and samples were vigorously shaken for 15mins to ensure complete mixing of chloroform and TRIzol®. Samples were incubated at RT for 3mins before centrifugation at 13,400 x g for 15mins at 4°C. The upper aqueous layer (the layer containing the RNA) was carefully removed, resuspended in 500µl of isopropanol and incubated at RT for 10mins, to precipitate RNA. Samples were centrifuged at 13,400 x g for 10mins at 4°C. Supernatant was removed and RNA was washed by resuspending the pellet in 1ml 70% ethanol, followed by centrifugation at 5,100 x g for 5mins at 4°C. The RNA pellet was then air dried for 5-8mins, before being resuspended in 30µl NF-H₂O and incubated at 60°C for 5-10mins to dissolve the RNA. All samples were stored immediately on ice to prevent RNA degradation. RNA yield and quality was then assessed using a NanoDrop® ND-1000 spectrophotometer (Thermo scientific, MA, USA), Agilent 2100 Bioanalyzer (Agilent Technologies) and agarose gels. RNA yield was not affected by IFN- α stimulation of PBMCs (Fig. 2.1). RNA quality was also unaffected (Fig. 2.2) and high quality RNA was successfully isolated from PBMCs from HCV patients (Fig. 2.3).

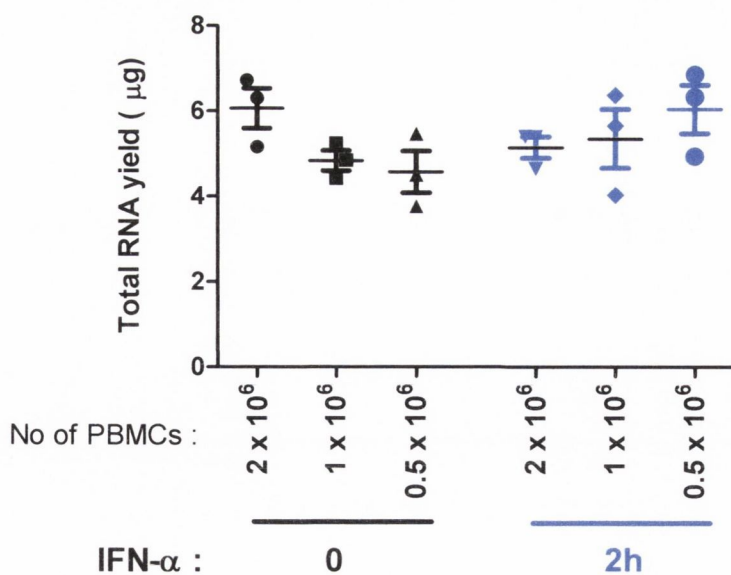


Figure 2.1. RNA yield from PBMCs with or without IFN-α stimulation

RNA yield was assessed using a ND-1000 NanoDrop® spectrophotometer. The NanoDrop® calculates RNA concentration based on Beer Lambert's Law, which states that $c = (A * e)/b$ where c is concentration in ng/µl, A is absorbance as calculated as $-\log(\text{Intensity}_{\text{sample}}/\text{Intensity}_{\text{blank}})$, e is the extinction coefficient for RNA and b is the path length in cm.

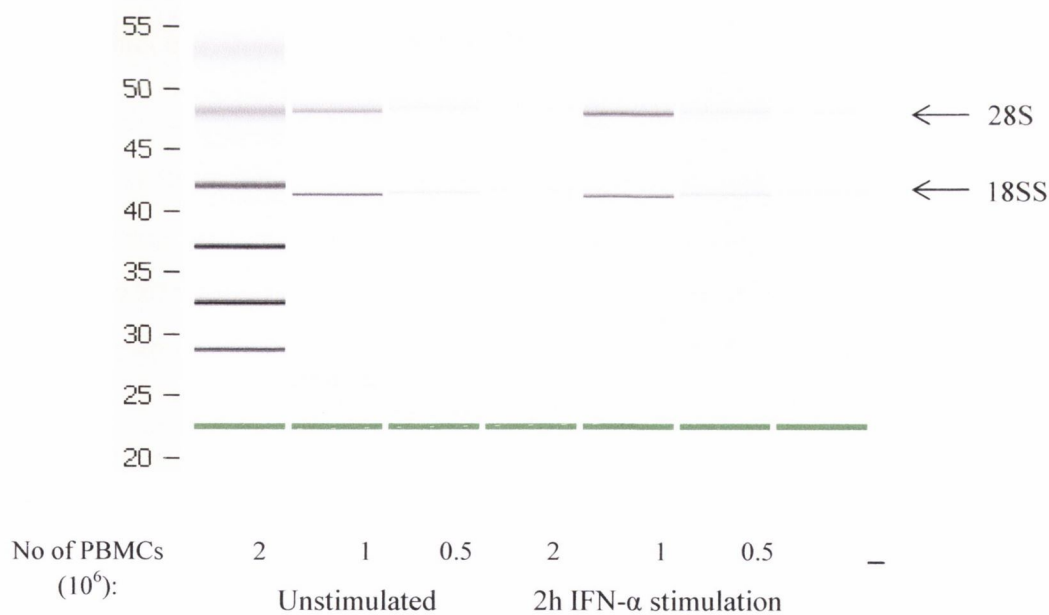


Figure 2.2. RNA quality: healthy PBMCs

A representative Agilent Bioanalyzer profile of RNA isolated from varying amounts of healthy PBMCs, with or without 2h stimulation with 1000IU IFN-α. RNA quality is assessed by the degradation visible as bands other than the 28S and 18S. The Agilent Bioanalyzer calculates an RNA Integrity Number (RIN) based on a numbering system from 1 to 10, with 1 being most degraded and 10 being most intact. All RNA from healthy PBMCs had a RIN > 8.

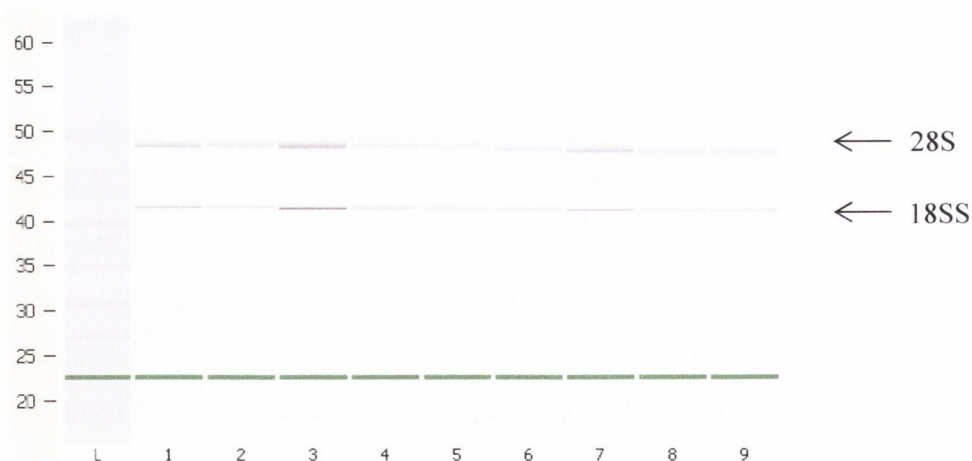


Figure 2.3. RNA quality: PBMCs from HCV patients

A representative Agilent Bioanalyzer profile of RNA isolated from PBMCs isolated from nine HCV infected patients. RNA quality is assessed by the degradation visible as bands other than the 28S and 18S. The Agilent Bioanalyzer calculates an RIN based on a numbering system from 1 to 10, with 1 being most degraded and 10 being most intact. All RNA from PBMCs from HCV patients had a RIN > 8.

2.3.7 cDNA synthesis and qRT-PCR

1µg of total RNA from each sample was reverse transcribed into cDNA with oligo-dT primers using an Omniscript RT Kit, using the following reaction: 2µl 10x reaction buffer, 8ng/µl Oligo dT, 0.25mM dNTP and 1µl Omniscript enzyme, added to 1µg RNA and made up to 20µl in NF-H₂O and incubated at 37°C for 1h. Following this, the enzyme was inactivated by heating the reaction to 95°C for 5min and cDNA was quantified using a NanoDrop® ND-1000 spectrophotometer. 40ng/µl dilutions were prepared for quantitative real time RT-PCR (qRT-PCR) reaction.

Each reaction was carried out in duplicate, in a total volume of 25µl with 2µl of cDNA (40ng/µl), 12.5µl SyBr green PCR master mix (Roche, Switzerland) and 10.5µl primer/H₂O. qRT-PCR was performed using a MX3000P® quantitative PCR system (Stratagene Corp, CA, USA) using the following cycling parameters: 95°C for 30sec, 60 °C for 1min and 72°C for 30sec followed by amplicon dissociation.

2.3.8 Primer design

Primers were designed to be intron-spanning where possible. This ensures that only cDNA made from mRNA extracted is being amplified by primers of interest and not possible genomic DNA contamination. Primers were designed using Primer Express® v3.0 software (Applied Biosystems). Primers with the lowest penalty score (i.e. the primers with closest match to primer criteria chosen) were used. To determine optimal primer concentration, concentrations of 100nM, 300nM, 600nM and 900nM were titrated using qRT-PCR. Amplification of a single PCR product was confirmed by analysing dissociations curves and visualisation on agarose gels. A full list of primers is given in Table 2.2.

2.3.9 GeNorm analysis

All gene amplifications for human samples were normalised to Ribosomal protein 15 (RPS15), which was selected as the most stably expressed gene across samples from a panel of potential normalisers: β -Actin, RPS15, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A protein (PPIA) β 2 microglobulin (B2M), glucuronidase β (GUSB) and RPS9 using GeNorm version 3.4 (Fig. 2.4).

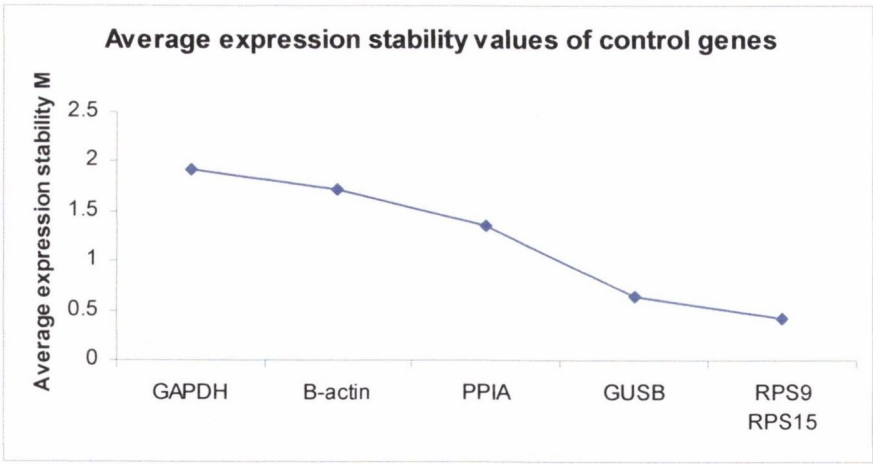


Figure 2.4. GeNorm analysis of reference genes

GeNorm analysis showing stability of reference genes across PBMCs, from healthy or HCV infected patients, stimulated with IFN- α . M values <1.5 are considered stably expressed genes.

2.3.10 Quantitative real-time polymerase chain reaction (qRT-PCR) data analysis

Data analysis was carried out using the $2^{-\Delta\Delta C_t}$ method [161]. C_t values were initially inputted into the formula $2^{\Delta C_t}$. A ratio of gene of interest relative to housekeeping gene was generating by normalising the gene of interest to a stable internal housekeeping control gene, as determined using geNORM analysis. If induction of a gene was being analysed, the ratio of the stimulated sample would be normalised to the ratio of the unstimulated sample; for sample induction values less than 1, -1 was divided by the induction value to calculate negative relative expression.

Gene	Forward	Reverse
β -actin	AGATGACCCAGATCATGTTTGAGA	CGTCACCGGAGTCCATCAC
B2M	GTGCTCGCGCTACTCTCTCTTT	GTCAACTTCAATGTCGGATGGA
CXCL10	CCAATTTTGTCACGTGTTGAG	GCTCCCCCTCTGGTTTTAAGGA
GAPDH	CCACCCATGGCAAATTCC	CAGCATCGCCCCACTTG
GUSB	ATCCGCACTGTGGCTGTCA	CCAGCGAAGCAGGTTGAAGT
IFN- α	GTACTGCAGAATCTCTCCTTTCTCCTG	GTGTCTAGATCTGACAACCTCCCAGGCACA
IFN- β	AACAAGTGCTCCTCCAAATTGC	GCAGTATTCAAGCCTCCCATTCT
MxA	GGTGGTGGTCCCCAGTAATG	ACCACGTCCACAACCTTGTCT
2'5'-OAS	GAAGCCCTACGAAGAATGTCAGA	TCGGAGTTGCCTCTTAAGACTGT
PKR	TCTCAGCAGATACATCAGAGATAAATTCT	AGTATACTTTGTTTCTTTTCATGTCAGGAA
PPIA	GCAAATGCTGGACCCAACAC	GCCATTCTGGACCCAAAG
RPS9	TGACCGGGTGGTTTGCTTA	ATACTCGCCGATCAGCTTCAG
RPS15	CGGACCAAAGCGATCTCTTC	CGCACTGTACAGCTGCATCA
STAT1	TGATGGCCCTAAAGGAACGTG	AGGAAAACGTGCGCCAGAGA
STAT2	CACACTATGCATGGTATCACAAACA	CTGAAGATTTCCATTGGCTCAGT
STAT3	GAGAAGGACATCAGCGTAAGAC	GCTCTCTGGCCGACAATACTTT
Trim 19	CCAACAACATCTTCTGCTCCAA	CTGGATCTCTGCGCTGATGTC
Trim 21	GGCCCTTTGCTGGGTATGT	CACTTCCAACCTTCTCAGCCAACCT

Table 2.2. Primers for qRT-PCR

2.3.11 Sample preparation for western blot

All cells for western blotting were harvested in radioimmunoprecipitation (RIPA) lysis buffer with protease inhibitors (20mM tris(hydroxymethyl)aminomethane (Tris), 150mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA) pH8, 1% v/v Triton X-

100, 0.1% w/v SDS, made up in dH₂O, supplemented prior to use with the protease inhibitors 5µg/ml aprotinin, 5µg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride (PMSF) and 1mM Na₃VO₄). For cell lysis, cells were incubated in the RIPA buffer on ice for 15mins. The cells were then centrifuged at 13,400 x g for 15mins. Cell supernatants, containing the proteins of interest, were harvested and stored at -20°C.

To determine protein concentration, a bicinchoninic acid (BCA) protein assay was performed. BCA reagent 2 was diluted 1:50 times in BCA reagent 1 and 200µl of this mix was added to a well of a 96 well plate. 3µl of the relevant sample was added and incubated at 37°C for 30mins. Protein concentration was determined using a BCA standard curve and all samples were made relative to the sample of lowest protein concentration. 5x blue juice sample (0.3M Tris, 50% v/v glycerol, 0.35M Sodium dodecyl sulphate (SDS), 10% v/v β-mercaptoethanol, pinch of bromophenol blue, made up in dH₂O) was added per sample and samples were boiled at 95°C for 5mins.

2.3.12 Western blotting

Reducing SDS-PAGE is a technique where a protein is denatured, i.e. it loses its quaternary and tertiary structure, and ran through a polyacrylamide gel using electrophoresis, which will separate proteins based on their molecular weight. Proteins can then be transferred to a polyvinylidene difluoride (PVDF) membrane where a protein of interest can be probed using a specific primary antibody for that protein. Secondary antibody, which will bind the primary antibody, is conjugated to an infrared dye is added. Protein can then be visualised on an Odyssey[®] Infrared Imaging System.

Pre-cast BIO-RAD Criterion[™] 4-12% Bis Tris gels were set up in gel apparatus with NuPAGE[®] MOPS SDS Running buffer. Gels were loaded with samples and molecular weight marker. Gels were initially run at 60V for 25mins until the protein had run through the stacking gel, the voltage was then increased to ~180V and samples were ran until the proteins reached the end of the gel.

Protein was transferred from the gel to a PVDF transfer using semi-dry transfer. Briefly, a PVDF membrane was soaked in methanol for 20secs to activate it, was rinsed in dH₂O for 2mins and transferred into a bath of transfer buffer (25mM Tris, 0.19M glycine, 20% v/v methanol, made up in dH₂O). Two blotting pads and the gel were also soaked in transfer buffer for at least 5mins. The semi-dry transfer was then set up as follows: blotting pad, PVDF membrane, gel, blotting pad. All were checked to confirm the absence of air bubbles. Transfer was run on a Biorad semi-dry transfer machine at 20V (500mA) for 90mins.

Membranes were blocked to prevent the non-specific binding of antibody in 5% milk solution (5% powdered milk in phosphate buffered saline (PBS, 1.37M NaCl, 82mM Na₂HPO₄, 15mM KH₂PO₄, 27mM KCL, made up in dH₂O) with 0.1% v/v Tween-20) for 30-60mins at RT. Blots were then incubated overnight at 4°C with primary antibody, diluted 1:1000 in 5% milk solution. The following day, relevant secondary antibody was added; this was at a dilution of 1:5000 in 5% milk solution, and was incubated rocking at RT for 30mins. Antibodies used are shown in Table 2.3. Proteins were analysed on an Odyssey[®] Infrared Imaging System.

Primary Antibody	Probing Dilution	Secondary Antibody
STAT1	1:1000	Goat anti-rabbit
STAT2	1:1000	Goat anti-rabbit
STAT3	1:1000	Goat anti-rabbit
NS2	1:1000	Goat anti-mouse
β-actin	1:1000	Goat anti-mouse

Table 2.3. Antibodies used for western blotting

2.3.13 Flow cytometry

To analyse phosphorylated STAT proteins, cells were fixed by adding an equal volume of pre-warmed Cytofix and incubated at 37°C in the dark for 10mins. Cells were washed by adding 1ml staining buffer (1% BSA, 0.01% sodium azide made up in PBS containing 0.1% Tween-20) and centrifuging at 290 x g for 5mins at 4°C. Supernatant was removed and cells were permeabilised by adding 1ml cold Phosflow Perm Buffer III and incubating on ice for 30mins. Cells were washed twice with stain buffer and

resuspended in 100µl stain buffer. 10µl pSTAT1 or 20µl pSTAT3 was added to relevant samples and incubated in the dark at RT for 30mins. Cells were washed once more with stain buffer and the final cell pellet was resuspended in 500ul stain buffer. All samples were acquired on a Dako Cyan flow cytometer and the data were analysed using Flowjo software (Tree Star Inc, OR, USA).

2.3.14 Plasmid transformation

Exogenous genetic material can be introduced into competent bacterial cells through the process of transformation. Transformation of plasmid DNA containing an origin of replication into competent bacterial cells allows for the rapid production of large quantities of plasmid DNA.

To transform DH5α cells, a competent *Escherichia coli* (E.Coli) bacterial strain, 2µl of plasmid DNA was added to 10µl of DH5α cells. Aliquoted DH5α cells were stored at -80°C and when required, thawed on ice. Plasmid DNA-DH5α mix was swirled gently and incubated on ice for 2mins. DH5α cells were then heat shocked by incubating at 42°C for 45secs; this promotes uptake of plasmid DNA. The mix was incubated on ice for a further 2mins. 100µl of pre-warmed Luria-Bertani (LB) was added and the LB mix was plated onto LB agar plates containing either 100µg/ml ampicillin or 12.5µg/ml tetracycline, depending on the vector being transformed.

To transform One Shot® Top10 chemically competent E.Coli cells, a 10µl aliquot of Top10 cells were thawed on ice and 2µl of DNA mix was added. This mixture was incubated on ice for 30mins before the bacterial cells were heat shocked at 42°C for 45secs. The reaction was incubated for a further 3mins on ice and 100µl pre-warmed LB broth. The reaction was shaken at 200rpms for 1h at 37°C, before being plated onto LB agar plates containing 100µg/ml ampicillin. LB agar plates were incubated for 12-16h at 37°C. Uptake of plasmid DNA was confirmed by the presence of bacterial colonies on agar plates. Positive and negative controls were used for each transformation.

2.3.15 Plasmid preparation and purification

Plasmid Mini-prep:

A single colony was added to 3mls LB broth containing 100µg/ml ampicillin and grown for 16h at 37°C while being shaken at 200rpms. 500µl of each culture was added to 500µl of glycerol and stored for future use at -80°C. The remainder of the cultures were mini prepped to isolate the plasmid DNA.

For purification of small amounts of plasmid DNA, Promega PureYield™ plasmid miniprep system (Promega Corporation, Madison, WI, USA) was used. 2.5ml of bacterial culture was centrifuged at 16,000 x g for 1min. Supernatant was discarded and cell pellet was resuspended in 600µl of NF-H₂O. 100µl Cell Lysis Buffer was added and the reaction was mixed by inverting 6 times. Within 2mins, 350µl pre-chilled Neutralisation Solution was added and the reaction was mixed thoroughly by inverting the tube. The reaction was centrifuged at 16,000 x g for 3mins before the supernatant was transferred to PureYield™ Minicolumn, which was then centrifuged at 16000 x g for 1min. The flow-through was discarded and 200µl of Endotoxin Removal Wash was added to the column. The column was centrifuged at 16000 x g for 1min before addition of 400µl Column Wash Solution and being centrifuged at 16000 x g for 1min. The minicolumn was transferred to a fresh, sterile 1.5ml eppendorf and 30µl of Elution buffer was carefully added to the column. After leaving this to stand at RT for 1min, the column was centrifuged at 16000 x g for 1min to elute plasmid DNA. Plasmid concentration was assessed using a NanoDrop® ND-1000 spectrophotometer.

Plasmid maxi-prep:

A single colony of transformed bacteria or 100µl of glycerol plasmid stock was added to 3mls LB broth and shaken at 200rpm at 37°C for 8h. This starter culture was then added to 150ml LB and shook at for a further 12-16h at 200rpm at 37°C. 500µl of culture was added to 500µl of glycerol and stored at -80°C. A Promega PureYield™ Plasmid Maxiprep System was used to isolate plasmid DNA. Cells were pelleted by centrifugation at 5000 x g for 10mins at RT and supernatant was discarded. Pellets were resuspended in 12ml Cell Resuspension Solution. 12ml Cell Lysis Solution was added and mixed by gently inverting 3-5 times followed by incubation at RT for 3mins. 12ml

Neutralisation Solution was added to the mixture, which was then inverted 10-15 times. Lysates was centrifuged at 14000 x g for 20mins at RT and added to a PureYield™ Clearing Column, which was placed on a PureYield™ Maxi Binding Column. A vacuum was applied until all liquid had passed through both columns. The clearing column was then removed. To wash the binding column, 5ml Endotoxin Removal Wash was added and a vacuum was applied until the liquid has passed through the column. 30ml of Column Wash solution was added and a vacuum was applied until the solution had passed through, after which vacuum was applied for an additional 5mins to ensure the column membrane was dry. Plasmid DNA was retrieved from the column by eluting in 1ml of NF-H₂O, which was added to the column, incubated at RT for 1min before vacuum being applied. Eluted DNA was retrieved in a sterile 1.5ml eppendorf. Plasmid concentration was assessed using a NanoDrop® ND-1000 spectrophotometer.

2.3.16 Agarose gels

PCR products were visualised on 1% Agarose dissolved in 1x TAE buffer (50x stock: 121g Tris base, 28.5ml glacial acetic acid, 18.6g Na₂EDTA.2H₂O; adjusted to 500mls) with Ethidium bromide (1:6000 dilution). Gels were ran at 120V for 20-40mins and visualised under UV light.

2.3.17 Cloning

To clone the E2-p7-NS2 polypeptide into the multiple cloning site of pcDNA3.1, primers were designed to amplify the E2-p7-NS2 region of HCV. Restriction sites for Hind III and Xho I were incorporated into the forward and reverse primers, respectively:

F: GCGGATC AAGCTT ATG GAAACCCACGTCACCGGGGGAAG

R: GTGGAGGTTGCTG GGTGGA CATCATCACCATCACCAC TAG CTCGAG
GATC

The PCR reaction was set up as follows: 5mM dNTPs, 10x Taq buffer, 2 μ M forward/reverse primer, 500ng HCV DNA construct, 2mM MgCl₂, 0.2 μ l platinum Taq; made up to 50 μ l with NF-H₂O. The insert was amplified using the following cycling parameters: 94°C for 2mins followed by 35 cycles of: 94°C for 30secs, 65°C for 30secs and 72°C for 1.5mins. The PCR product was ran on an agarose gel and the product was excised and removed from the gel using a Promega gel clean up kit. The PCR product, along with pcDNA3.1, were digested using the following reaction: 1 μ l HindIII, 1 μ l Xho I, 0.7 μ l 100x bovine serum albumin (BSA), 6 μ g DNA and 7 μ l buffer 2 (NEB), made up to 70 μ l in NF-H₂O, incubated at 37°C for 1h. The reactions were visualised on an agarose gel and digested DNA was extracted from the gel using a Promega Wizard® SV Gel and PCR Clean-Up System. For ligation, a 1:3 vector:insert ratio was used. This resulted in 108ng of digested insert being ligated into 100ng of digested pcDNA3.1 in the following reaction: 2 μ l DNA ligase (NEB), 6 μ l 10x reaction buffer, made up to 60 μ l in NF-H₂O. This reaction was incubated at 16°C for 1h before being transformed into Top10 competent cells. Following transformation, 10 colonies were chosen for mini-prep to confirm presence of ligated insert. Prepared DNA was sent for sequencing to confirm the presence of ligated insert. Following confirmation of insert, maxi preps from 100 μ l glycerol stocks grown up in LB broth were used for large-scale preparation of plasmid DNA.

2.3.18 Gel Clean up

To extract DNA from agarose gel, a Promega Wizard® SV Gel and PCR Clean-Up System was used. 10 μ l of Membrane Binding Solution was added per 10mg of agarose gel. The mixture was vortexed and incubated at 60°C for up to 10mins, until the gel had dissolved. The gel mixture was added to a SV Minicolumn and incubated at RT for 1min. The column was centrifuged at 16000 x g for 1min, the flow-through was discarded and 700 μ l Membrane Wash Solution was added. This was centrifuged at 16000 x g for 1min, flow-through was discarded and the column was centrifuged again for 5min at 16000 x g to dry the membrane. The column was placed on a sterile 1.5ml eppendorf, 50 μ l NF-H₂O was carefully added to the membrane and incubated at RT for

1min. To elute DNA, the column was centrifuged at 16000 x g for 1min. DNA concentration was assessed using a NanoDrop[®] ND-1000 spectrophotometer.

2.3.19 Luciferase reporter gene assay

As a measurement of IFN- α activity, Firefly luciferase reporter gene assays were used where the promoter of interest [either ISRE or γ -activated sequence (GAS)] is upstream of a luciferase gene and when stimulated, bioluminescent activation can be measured. As an internal transfection control, the constitutively active plasmid TK-Renilla was co-transfected with all luciferase vectors.

Per well, cells were transfected with 60ng of either ISRE-luciferase or GAS-luciferase reporter gene plasmids, 20ng TK-Renilla and 150ng of EV (either pBR322 or pcDNA3.1), HCV or cloned vectors as indicated. Cells were treated with IFN- α as indicated. To harvest the cells, media was removed by inversion and lysed for 15mins using 50 μ l Passive Lysis Buffer with vigorous shaking. To assay firefly luciferase activity, 40 μ l luciferase assay mix (20mM tricine, 1.07mM (MgCO₃)₄Mg(OH)₂.5H₂O, 2.67mM MgSO₄, 0.1M EDTA, 33.3mM DTT, 270mM coenzyme A, 470mM luciferin, 530mM ATP) was added to 20 μ l cellular lysates. To quantify Renilla luciferase activity, 40 μ l of 2 μ g/ml Coelenterazine (diluted from stock in 1x PBS) was added to 20 μ l of lysates. Luminescence was read using a Reporter microplate luminometer. All firefly luciferase values were normalised relative to the Renilla activity for that sample and expressed as fold induction over unstimulated EV.

2.3.20 Short hairpin RNA (shRNA) and small interfering RNA (siRNA)

For siRNA, 60pmol of scrambled or native STAT3 siRNA was made up to 100 μ l in siRNA transfection media. A 2:1 ratio of siRNA transfection reagent to siRNA was made up in 100 μ l siRNA transfection media. These two solutions were mixed gently together and incubated in the dark for 15-45mins. Huh-7 cells (1x10⁵/well/6 well plate) or PBMCs (5x10⁶/well/12 well plate) were washed twice with transfection media, with a final volume of 800 μ l transfection media left on the cells. 200 μ l siRNA mix was then

added to cells and incubated for 5-7h. 1ml of DMEM supplemented with 20% FCS was then added to the cells. After 18-24h, media was removed and DMEM supplemented with 10% FCS was added to cells. 36h later, cells were stimulated as indicated and harvested in 1ml of TRIzol® for mRNA analysis.

For shRNA, Huh-7 cells, 1×10^5 /well/6 well plate, were transfected with 500ng of scrambled, STAT1 or STAT3 shRNA, or both together using lipofectamine. Media was changed after 24h. 24h after media was changed, cells were stimulated as indicated and harvested in 1ml of TRIzol® for mRNA analysis.

2.3.21 Bioinformatics

ClustalW:

The ClustalW multiple sequence alignment 2.0 tool (<http://www.ebi.ac.uk/tools/clustalw2>) was used to align multiple protein sequences to compare amino acid similarities of the sequences [163]. This software attempts to find the best alignment match from input sequences based on the Gonnet substitution matrix, a matrix that gives a score for amino acid mismatches, based on a broad range of protein sequences.

Fuzzpro:

To identify potential motifs within protein sequences, FuzzPro, an EMBOSS software programme (www.emboss.org), was used. Short protein sequence motifs input into this software as regular expressions, which can be of varying length, with amino acids being represented by their IUPAC one letter codes, unknown amino acids represented by x and varying lengths can be represented by numbers (e.g. C-[MF]-x(2,4)-L would search for a pattern starting with a cysteine residue, followed by either a methionine or a phenylalanine residue, followed by 2-4 unknown residues and finally a leucine residue).

2.3.22 Statistics

To test differences in values between two independent patient sample groups, non-parametric Mann-Whitney U tests were used. Non-parametric tests were used for patient samples as normal Gaussian distribution could not be assumed with the low patient numbers being assessed; therefore use of parametric statistical tests could have resulted in inaccurate analysis of the data. For paired patient sample analysis, Wilcoxon matched pairs analysis was used. To analyse differences within a cell line, a Student's T test was used as normal distribution was assumed within a cell line. All were calculated using Graphpad Prism® 5 software. To analyse the difference in the extent of variability between two groups of individuals, a non-parametric Levene's test was used. This was done using Statistical software package SPSS, version 16.0 to rank the data, calculate the mean rank value and subtract that from each rank value. A one way analysis of variance was then used to test the variance between the groups.

p-values <0.05 were considered statistically significant.

Chapter 3

**HCV degrades STAT3 via the proteasome:
identification of inhibitory peptides against
viral targets that restore IFN- α
responsiveness**

Chapter 3 HCV degrades STAT3 via the proteasome: identification of inhibitory peptides against viral targets that restore IFN- α responsiveness

3.1 Introduction

Viral recognition by the innate immune system results in the systemic induction of anti-viral responses within the host, most notably through the activation and rapid production of type I IFNs, which switch on potent anti-viral defences capable of directly limiting viral replication and activating the adaptive immune response. Type I IFNs activate STAT transcription factors, which dimerise and translocate to the nucleus, binding specific promoter elements and upregulating expression of ISGs. Two common promoter elements activated by IFN- α are ISRE and GAS. IFN- α activates STAT homo- and hetero-dimers and specificity for either the ISRE or GAS promoter is dependent on the STAT dimer formed. Some ISGs only contain a promoter element for one, whereas other contain promoter elements for both [164].

50-90% of those infected with HCV fail to naturally clear the virus [2], therefore it is a reasonable hypothesis that HCV codes for an immunomodulator capable of subverting type I IFN responses. Up until recently, the only therapy for treating HCV was IFN- α , which should in theory allow the rapid inhibition of HCV replication. However, up to 60% of patients fail to respond to this treatment, particularly genotype 1 infected patients, further supporting the hypothesis that type I IFN is a target for HCV and that the IFN- α pathway in some individuals is susceptible to HCV targeted attack. As IFN- α signals through the JAK-STAT pathway, and it had been previously been shown that HCV can target STAT1 [165], we were particularly interested in investigating the effect of HCV on STAT transcription factors and the consequence on ISG induction.

3.1.1 HCV and STAT proteins

STAT1 and STAT2 are activated by IFN- α and form the ISGF-3 complex with IRF9. The ISGF-3 complex binds to specific promoter elements, resulting in the upregulation of over 500 anti-viral genes, which exhibit diverse roles such as anti-viral immunity and control of cell growth [3]. In 2005, Lin et al., reported that HCV could target STAT1 for degradation in a Huh-7 hepatocyte cell line. They found that STAT1 was degraded in a proteasome-dependent manner, as the proteasomal inhibitor MG132 could recover STAT1 protein expression [166]. Later studies found that the NS5A protein from HCV also decreased STAT1 protein expression [166,167]. STAT1 expression in the liver has been linked with treatment response to IFN- α therapy. Liver biopsies from HCV patients showed an impaired ability for activated STAT1 to bind DNA following activation, due to an upregulation of the negative regulator protein phosphatase 2A (PP2A) [151]. PP2A upregulation was later shown to be caused by HCV replication on the ER, inducing stress and calcium release, which promotes PP2A expression [168].

Although STAT1 and STAT2 are seen as the classical proteins activated by IFN- α , other STAT proteins are also involved in IFN- α signal transduction [150,167]. STAT3 is involved in many cellular processes such as proliferation and growth factor signalling, although its role in IFN- α signalling remains controversial. We found that both STAT1 and STAT3 expression was significantly reduced in circulating leukocytes from chronically infected HCV patients (Data provided by Dr. Nigel Stevenson). Altered STAT3 protein expression by HCV could be reversed using the proteasomal inhibitor MG132, suggesting that HCV targets STAT3 for proteasomal degradation (Fig. 3.1). We therefore sought to determine how HCV could be targeting STAT3 protein for proteasomal degradation. Specific targeting of STAT3 protein expression by HCV may be due to an important, unknown role for this protein in the IFN- α anti-viral response.

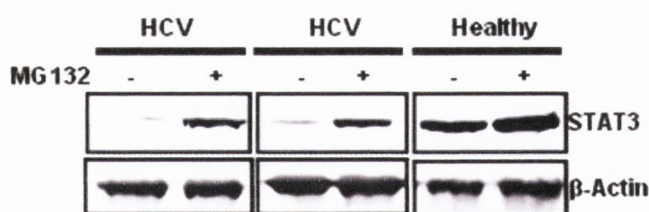


Figure 3.1. Proteasomal inhibition recovers STAT3 expression in PBMCs from HCV patients

Western blot analysis of STAT3 protein levels in the PBMCs of HCV infected patients, with or without the proteasomal inhibitor MG132.

3.1.2 Viral targeting of STAT proteins for degradation via the proteasome

Targeting proteins for degradation via the proteasome is a proteolytic cellular process that recycles redundant or misfolded proteins, by labelling them with poly-ubiquitin chains, to produce free amino acids. Ubiquitin is a small molecule that is post-translationally attached to target proteins via an isopeptide bond, leading to two possible outcomes. If a single ubiquitin molecule is attached, then the tagged protein will be involved in non-proteolytic events such as signal transduction; whereas if the protein is tagged with a polyubiquitin chain via a lysine 48 residue, that protein is targeted to the 26S proteasome for degradation [169]. The 26S proteasome recognises proteins with a polyubiquitin chain attached, and subsequently mediates their degradation through its protease activity [169].

3.1.3 Ubiquitination and proteasome-mediated degradation

The attachment of ubiquitin molecules to a target protein involves three enzymes, an activating E1 enzyme, a conjugating E2 enzyme and a ligase E3 enzyme (Fig. 3.2). Two E1 enzymes are found in humans that activate the C terminus of the ubiquitin molecule [170]. The activated ubiquitin molecule is then transferred to the conjugating E2 enzymes, for which there are thought to be approximately 30 genes identified in humans. E2 conjugating enzymes transfer ubiquitin molecules to the E3

ubiquitin ligase. Over 600 E3 ligases have been identified in humans [171] and they bind the target substrate to mediate covalent attachment of the ubiquitin molecules [172]. E3 ligases link the ubiquitin process to the specific protein targeted for degradation and hundreds of these ligases have been identified. They are involved in diverse cellular processes and malfunction of their regulation has been implicated in many diseases, such as cancers [173]. The specificity of E3 ligases for target substrates has made them attractive targets for diseases such as cancers, where proteasomal inhibition would enhance responsiveness to treatment but avoiding the severe side-effects associated with the clinical administration of general proteasome inhibitors [174].

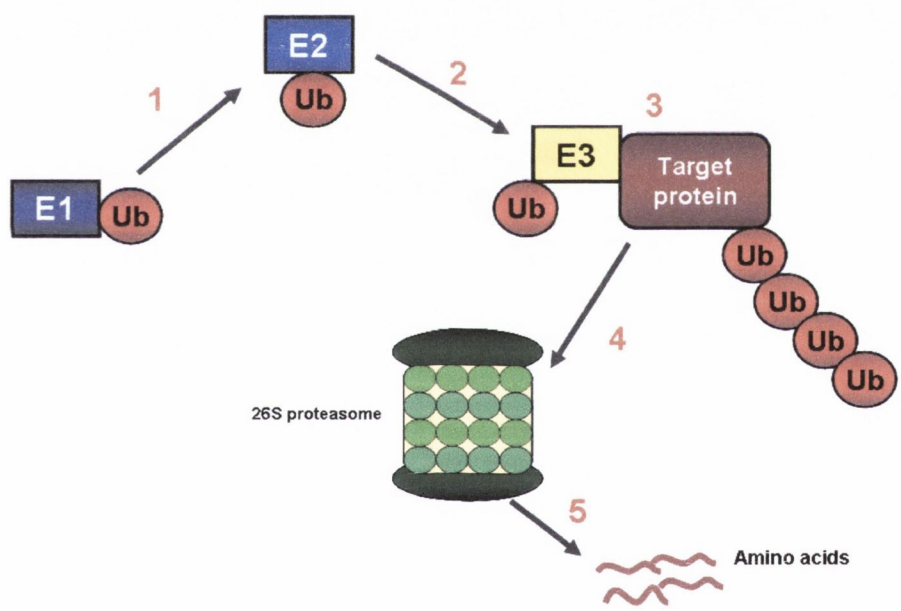


Figure 3.2. Polyubiquitination of target proteins results in protein degradation via the proteasome

1. An E1 enzyme catalyzes the transfer of an ubiquitin molecule to an E2 enzyme. 2. The E2 enzyme catalyzes the transfer of the ubiquitin molecule to the E3 adapter ligase. 3. The E3 adapter polyubiquitinates the target protein on a lysine 48 residue. 4. The polyubiquitinated target protein is recognized by the 26S proteasome. 5. The 26S proteasome degrades the target protein, releasing free amino acids into the cell.

Some E3 ligases, such as SOCS proteins and Von Hippel-Lindau (VHL) tumour suppressor protein, bind to the E2 complex via elongin and cullin (cul) proteins. E3 ligases containing elongin B and elongin C binding sequences (known as a BC box) also bind to either cul-2 or cul-5 molecule [172]. Cul-2 binding E3 ligases bind a VHL box, named after VHL tumour suppresser protein, the first protein described to contain this motif [175]. One known function of VHL is to induce proteasomal degradation of hypoxia inducible factor 1 α (HIF-1 α), a transcription factor that regulates cellular responses during hypoxia. HIF-1 α regulates the expression of genes involved in angiogenesis and erythropoiesis, promoting their upregulation during hypoxia, whereas under non-hypoxic conditions, VHL degrades HIF-1 α via the proteasome [176,177]. Mutations in the VHL tumour suppressor gene causes VHL disease, an inherited cancer, which occurs due to the failure of VHL tumour suppressor to function as an E3 ligase and degrade HIF-1 α , reflecting the aberrant effects associated with deregulation of the ubiquitination and proteasomal degradation system [178]. Cul-5 binding E3 ligases mediate degradation via a SOCS box, named after SOCS proteins [175]. SOCS proteins regulate cytokine signalling through their E3 ligase activity and control type I IFN signalling by targeting the IFNAR and JAK proteins for proteasomal degradation [179].

3.1.4 Virally encoded E3 ligases with VHL- and SOCS- boxes

Many viruses have hijacked this system by encoding proteins with VHL- and SOCS- boxes, allowing them to act as E3 ligases and target proteins involved in anti-viral immunity for degradation. These viral E3 ligases can recruit elongin and cul proteins, allowing them to polyubiquitinate target substrates. HIV vif protein contains a SOCS box which recruits elongin C, along with elongin B and cul-5, and targets the anti-viral protein APOBEC3G for proteasomal degradation [180]. Adenovirus encodes E4orf6, a protein that also contains a SOCS box, allowing the virus to induce proteasomal degradation of p53, a protein that suppresses cell growth [181]. Many viruses have been shown to specifically target STAT proteins for degradation to evade type I IFN responses. Paramyxoviruses encode a V protein which allows them to degrade many STATs including STAT1, STAT2 and STAT3, although this is dependent

on cul-4 and therefore independent of a VHL- or SOCS- box [182]. One virus that degrades STAT proteins using a VHL-box is RSV. RSV encodes a protein, NS1, which allows it to bind to elongin C and cul-2 and target STAT2 for proteasomal degradation [147]. Bioinformatic analysis studies from our lab revealed that HCV may encode its own E3 ligase similar to that seen in RSV NS1. A multiple sequence alignment of VHL tumour suppressor protein and RSV NS1 shows that there is a putative binding site in the p7 protein of HCV genotype 1 (Fig. 3.3).

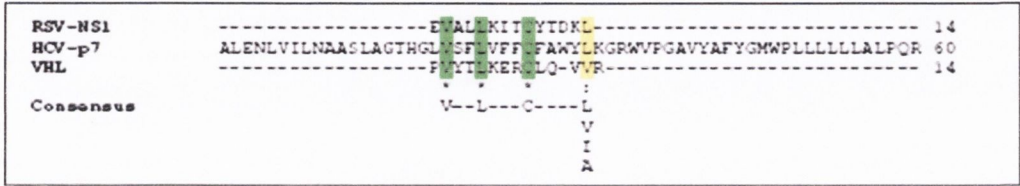


Figure 3.3. The elongin C binding site in RSV NS1, HCV p7 and VHL

ClustalW multiple sequence alignment of two elongin C binding proteins, NS1 from RSV and the VHL tumour suppressor protein, with the p7 protein from HCV. Amino acids known to be involved in elongin C binding are conserved within the p7 protein of HCV.

Therefore, we propose that HCV functions as an E3 ligase to allow it to mediate the degradation of STAT proteins and evade innate anti-viral responses activated by IFN- α .

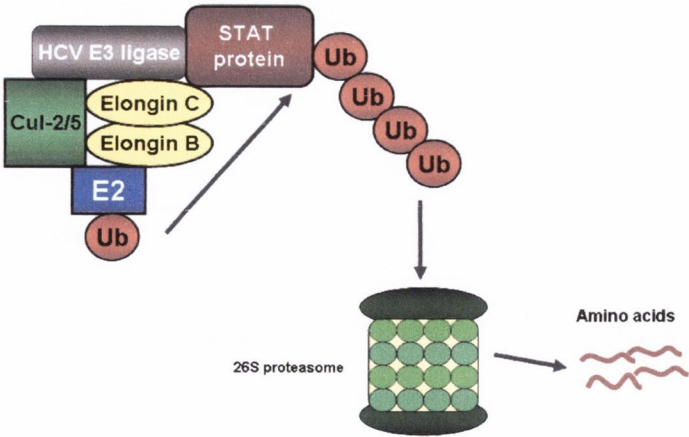


Figure 3.4. Proposed model for HCV-mediated STAT proteins degradation

HCV contains a putative elongin C binding site that may allow it to target STAT proteins for proteasomal degradation, similar to other viral proteins that encode elongin C binding sites.

3.1.5 Aims

The aim of this study was to establish the mechanism by which HCV targets STAT3 protein expression. We also wanted to examine if the absence of STAT3 had a consequence on IFN- α induced anti-viral immunity. We aimed to determine the molecular mechanism used by HCV to degrade STAT3 using bioinformatic analysis to identify the regions of the HCV genome with motifs similar to those known to act as elongin C binding E3 ligases. This would allow us to test whether specifically targeting the identified viral regions that mediate protein degradation could restore STAT3 protein expression and recover IFN- α signalling, and therefore represent a possible novel therapeutic intervention during HCV infection.

3.2 Results

3.2.1 STAT1 and STAT3 protein expression is reduced by HCV

To examine the effects of HCV protein expression, we use a hepatocyte cell line (Huh-7s) stably expressing a T7 polymerase and a cDNA construct, which under control of a T7 promoter, expresses all the structural and NS proteins of HCV. Following transfection of Huh-7 cells with the HCV construct, both STAT1 and STAT3 protein expression was starkly reduced, whereas STAT2 was not affected (Fig. 3.5). HCV protein expression was confirmed by probing for NS2.

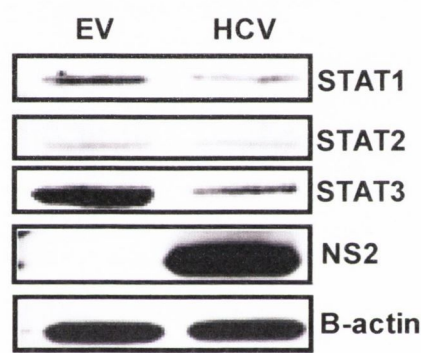


Figure 3.5. STAT1 and STAT3 protein expression is reduced in Huh-7 cells expressing HCV

Huh-7s were transfected with either pBR322 as a control (EV) or HCV for 16 hours, before being lysed and analysed by western blotting. Antibodies against STAT1, STAT2, STAT3, NS2 and β -actin were used.

3.2.2 STAT1 and STAT3 mRNA is unaffected by HCV expression

To assess whether HCV was altering STAT1 and STAT3 protein expression by affecting mRNA levels of these proteins, qRT-PCR was used to measure the gene expression level for both (Fig. 3.6). STAT2 was also measured as a control. HCV had no affect on STAT1, STAT2 or STAT3 mRNA levels compared to cells transfected with EV control.

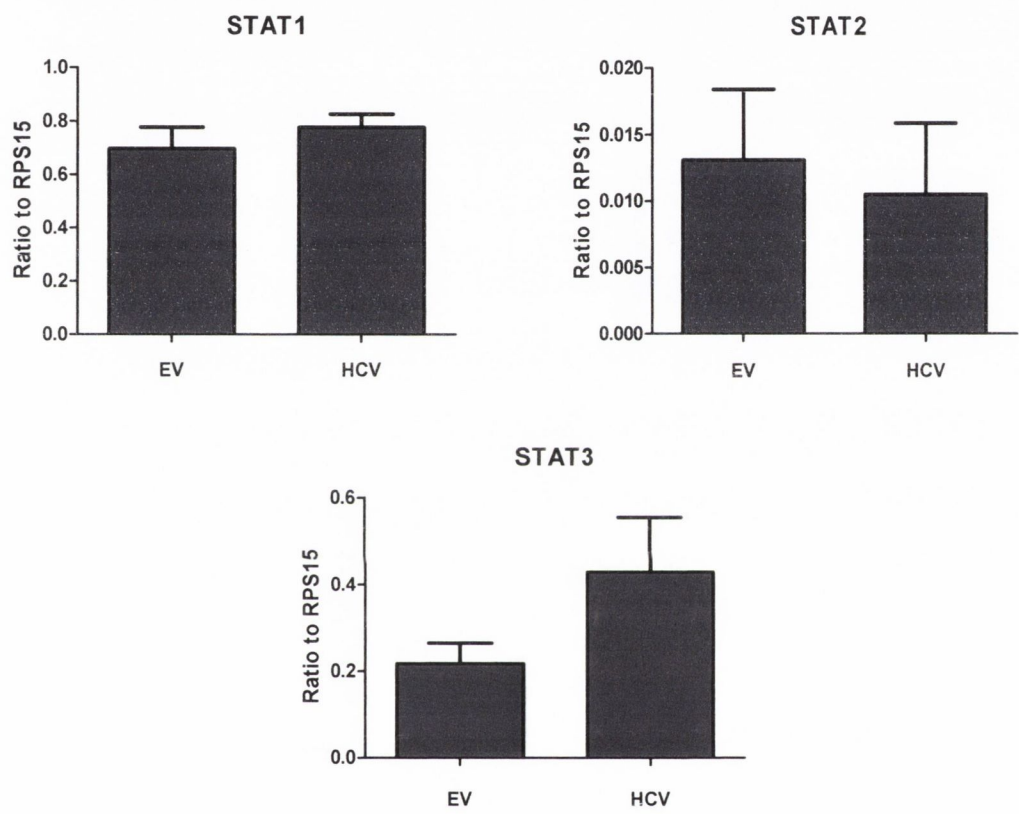


Figure 3.6. HCV expression does not reduce STAT1, STAT2 and STAT3 mRNA expression

qRT-PCR was used to analyse the expression of STAT1, STAT2 and STAT3 mRNA in Huh-7 hepatocytes transfected with either EV (pBR322) or HCV for 16h (n=3). All samples were normalised to endogenous RPS15 expression.

3.2.3 IFN-α stimulation upregulates ISGs in Huh-7 cells

As HCV can alter expression of STAT1 and STAT3 protein in Huh-7 cells, we sought to analyse the consequence of reduced expression of these transcription factors on IFN-α induced ISG expression. Initially, we determined the optimal time point for ISG expression in these cells. We chose PKR, OAS and MxA as our read-out for ISG induction as these are three well-studied IFN-α induced ISGs (Fig. 3.7). By 2h post IFN-α stimulation, PKR, OAS and MxA mRNA expression was significantly upregulated compared to unstimulated Huh-7s ($p<0.05$). This upregulation was highly significant for PKR expression 4h post stimulation ($p<0.01$). 6h IFN-α stimulation upregulated all 3 ISGs with high significance compared to unstimulated cells ($p<0.01$).

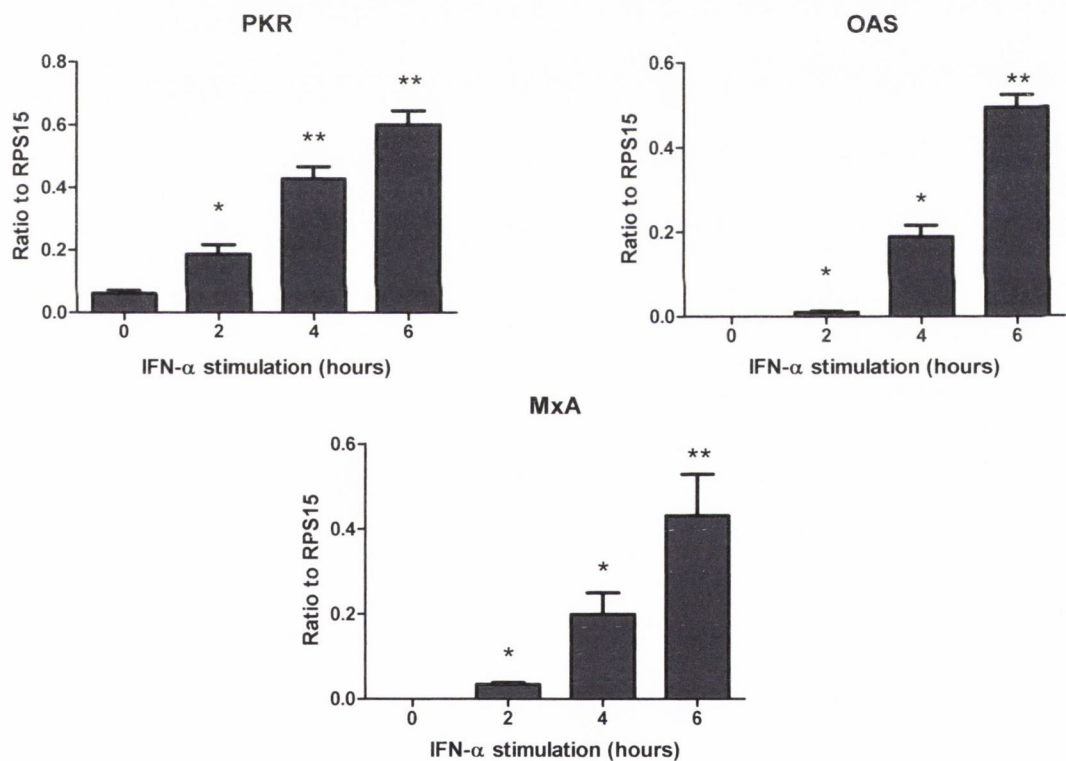


Figure 3.7. IFN-α upregulates ISG expression in Huh-7 hepatocytes

Huh-7 hepatocytes were stimulated with 1000IU IFN-α for 2, 4 and 6 hours before mRNA for PKR, OAS and MxA was analysed using qRT-PCR. All samples were normalised to endogenous RPS15 expression. N=3, statistical differences analysed using Student's T test compared to unstimulated; * $p<0.05$, ** $p<0.01$.

3.2.4 HCV blocks IFN- α induced gene expression in Huh-7 cells

In Huh-7 hepatocytes, expression of HCV leads to decreased expression of STAT1 and STAT3 protein. As these transcription factors are important for IFN- α signal transduction, we examine the effect of HCV expression on ISG induction. Huh-7 cells were transfected with HCV and stimulated with IFN- α . We found that transfection with HCV significantly reduced ISG induction (Fig. 3.8). Induction of both PKR and MxA were both significantly reduced in the presence of HCV ($p<0.05$); OAS induction was also reduced, although not significantly ($p<0.07$).

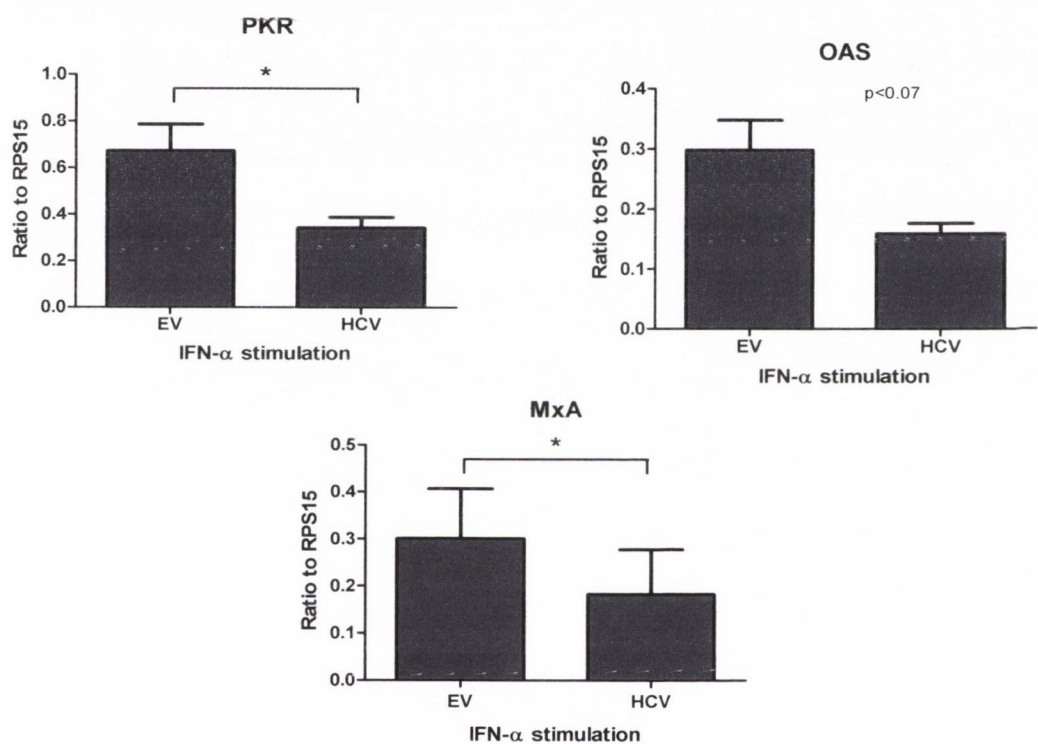


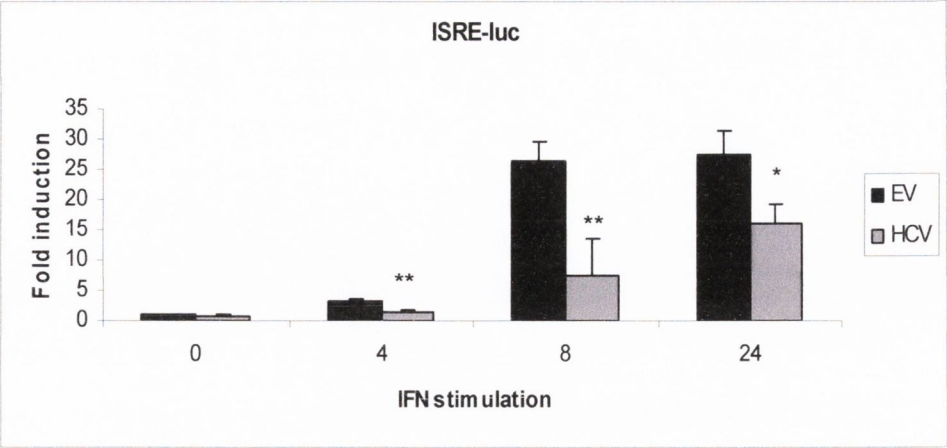
Figure 3.8. HCV blocks ISG induction in Huh-7 hepatocytes

Huh-7 hepatocytes were transfected with EV or HCV for 16h and stimulated with 1000IU IFN- α for 4h. qRT-PCR was used to analyse mRNA expression of PKR, OAS and MxA ($n=4$). All samples were normalised to endogenous RPS15 expression. Statistical differences analysed using Student's T test, * $p<0.05$.

3.2.5 HCV expression significantly inhibits ISRE and GAS induction

As we found that the induction of the ISGs PKR, OAS and MxA was significantly decreased by HCV expression, we sought to assess the effect of HCV expression on the activation of ISRE and GAS promoters. We used firefly luciferase vectors under the control of the ISRE or GAS promoter sequences, which when activated by a suitable stimulus, induce quantifiable luciferase expression. Huh-7s transfected with either ISRE or GAS luciferase vectors were also transfected with either EV control or HCV and stimulated with IFN- α over a time course (Fig. 3.9A and 3.9B respectively). IFN- α strongly activated both ISRE-luc and GAS-luc, after 8h and 24h stimulation. However, expression of HCV significantly inhibited activation of ISRE and GAS promoter activation by IFN- α . For ISRE-luc, HCV inhibition of induction was highly significant after 4 and 8h stimulation ($p<0.01$) and significantly inhibited after 24h stimulation ($p<0.05$). HCV significantly inhibited GAS-luc induction after 8h IFN- α stimulation ($*p<0.01$).

(A)



(B)

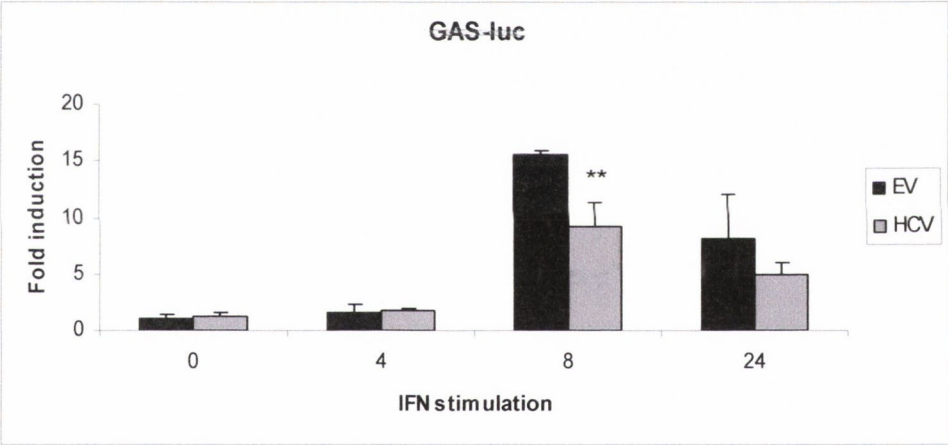


Figure 3.9. HCV inhibits ISRE and GAS promoter activity

Huh-7 cells were transfected with firefly reporter plasmid, either (A) ISRE-luciferase or (B) GAS-luciferase, TK-Renilla reporter plasmid and either pBR322 or HCV. The cells were stimulated with 1000IU IFN- α for 0, 4, 8 or 24h and then assayed for ISRE or GAS activation. N=3; statistical differences analysed using Student's T test, * p<0.05 or ** p<0.01 compared to EV.

3.2.6 Endogenous ISG expression is dependent on STAT1 and STAT3 expression

Huh-7s expressing HCV significantly reduces both STAT1 and STAT3 protein expression and subsequently fail to induce ISGs following IFN- α stimulation, therefore we aimed to determine the relevant contribution of STAT1 and STAT3 to endogenous ISG expression within Huh-7 cells. We used shRNA against STAT1 or STAT3, and analysed expression of PKR, OAS, MxA and CXCL10, a chemokine upregulated by IFN- α (Fig. 3.10). We found that cells transfected with either STAT1 or STAT3 shRNA, and both together, had decreased endogenous expression of PKR, OAS and MxA, but not of CXCL10, compared to cells transfected with control shRNA.

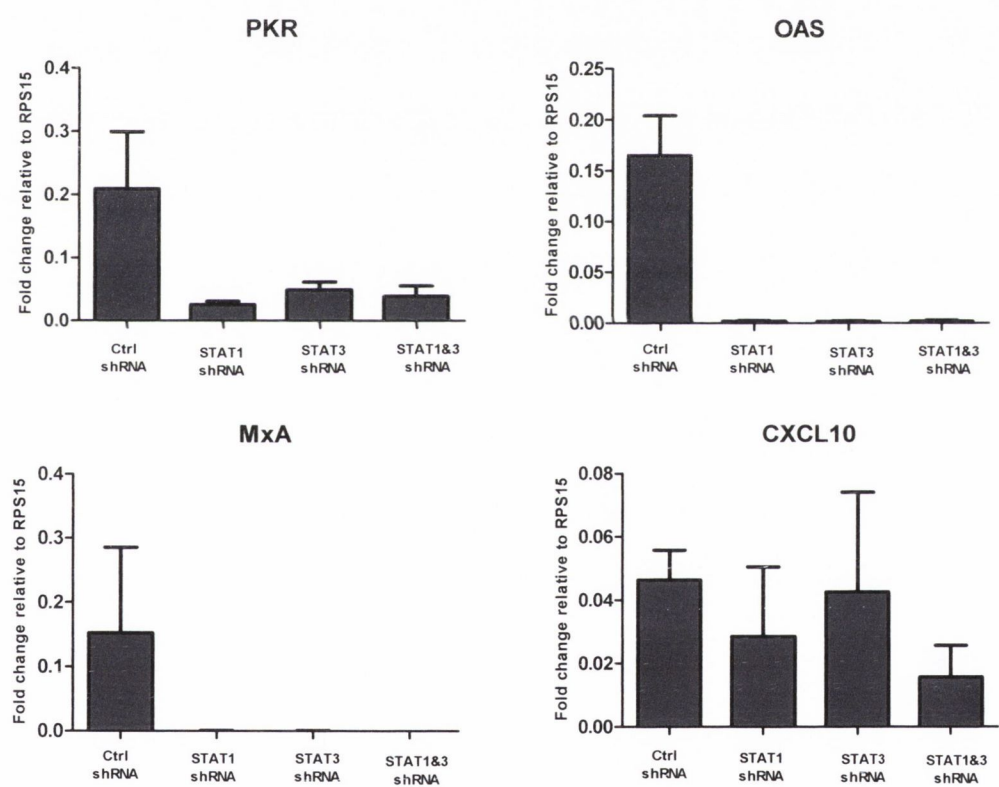


Figure 3.10. Endogenous ISG expression is dependent on STAT1 and STAT3 expression

Huh-7 cells were transfected with either control shRNA, STAT1 or STAT3 shRNA, or both together for 48h. Endogenous levels of PKR, OAS, MxA and CXCL10 were measured by qRT-PCR. All samples were normalised to RPS15 housekeeping control.

N=3

100

3.2.7 ISG induction is dependent on STAT1 and STAT3 expression

Having observed that STAT1 and STAT3 expression is necessary for endogenous expression of ISGs, we investigated the role of STAT1 and STAT3 in ISG induction following IFN- α stimulation. Huh-7s cells were transfected cells with either STAT1 or STAT3 shRNA, or both together, stimulated with IFN- α for 2h, before measuring ISG induction (Fig. 3.11). We found that STAT1 and STAT3 shRNA, either alone or in combination, decreased induction of PKR, OAS and MxA. Interestingly, CXCL10 induction was also reduced with shRNA against STAT1 or STAT3.

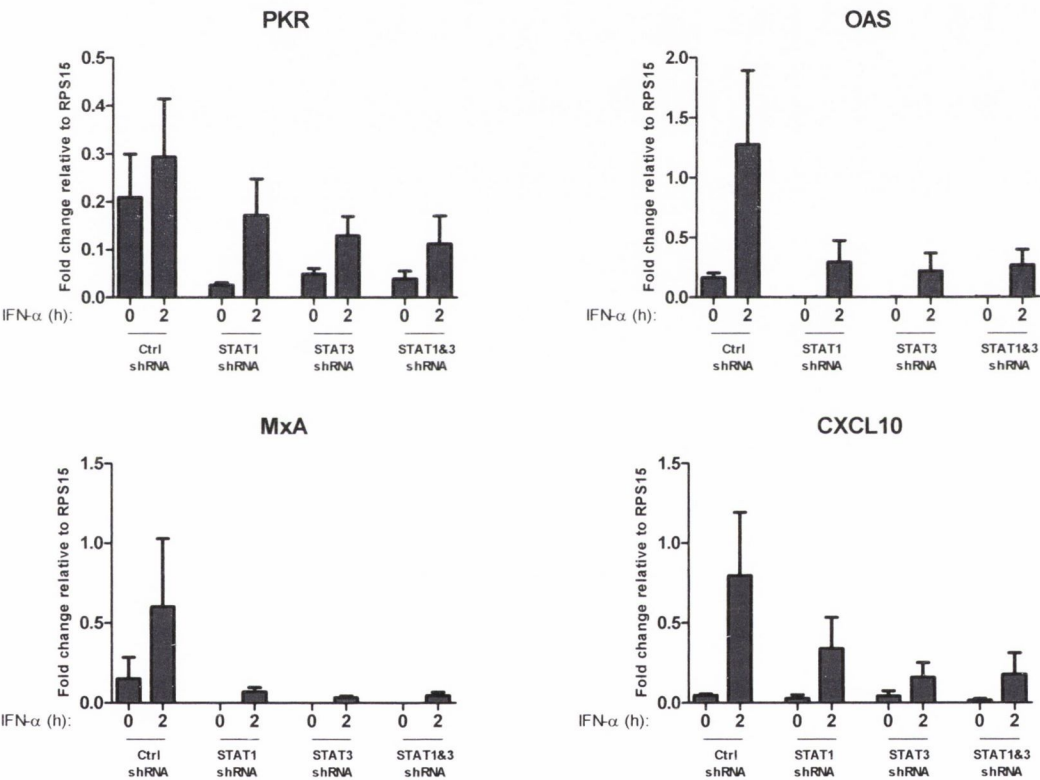


Figure 3.11. ISG induction is dependent on STAT1 and STAT3

Huh-7 cells were transfected with control shRNA, STAT1 shRNA, STAT3 shRNA or both together for 48h before being stimulated with 1000IU IFN- α for 2h. qRT-PCR was used to measure PKR, OAS, MxA and CXCL10 mRNA expression. All samples were normalised to RPS15 housekeeper control. N=3

3.2.8 STAT3 is required for ISG induction

As the role for STAT1 is well established in ISG induction, we sought to further confirm the contribution of STAT3 in ISG induction using an alternative method of gene inactivation: siRNA against STAT3 (Fig. 3.12). Huh-7s treated with STAT3 siRNA showed significantly reduced STAT3 gene expression ($p<0.01$). Endogenous levels of PKR and MxA, and also induction of these genes following stimulation, was decreased when STAT3 expression was reduced using STAT3 siRNA.

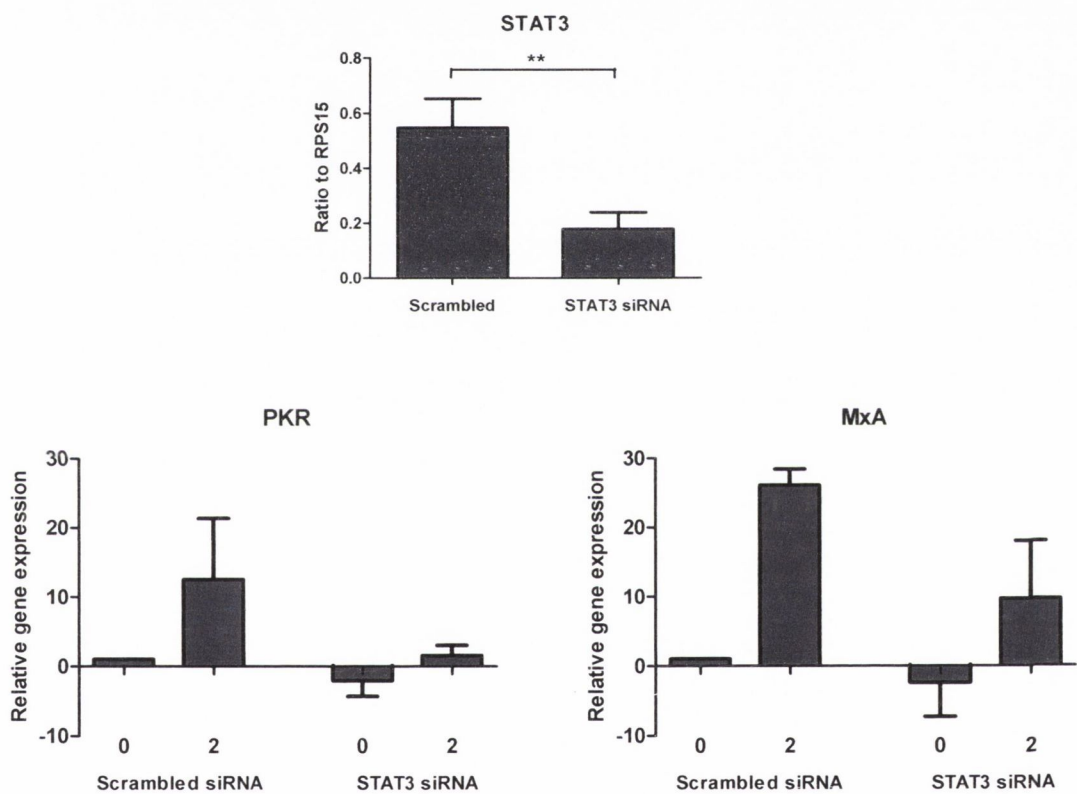


Figure 3.12. STAT3 siRNA reduces ISG induction in Huh-7 hepatocytes

qRT-PCR analysis of STAT3 ($n=6$), PKR and MxA mRNA expression (both $n=3$) in Huh-7 hepatocytes transfected with scrambled or STAT3 siRNA, $\pm$ 1000IU 2h IFN- α . All samples are normalised to RPS15 housekeeping control and values are relative to unstimulated scrambled control. statistical differences analysed using Student's T test, $**p<0.01$.

3.2.9 The elongin C binding site is conserved in genotype 1 of HCV

To further analyse the putative elongin C binding site within the p7 protein of HCV, consensus sequences for different HCV genotypes were generated by retrieving sequences of the p7 protein from known genotypes from the Los Alamos HCV Sequence Database, where each sequence is from a different patient [147,175]. Consensus sequences were generated, using the consensus maker tool available within the database, for genotype 1a (325 seqs), 2 (30 seqs), 3 (10 seqs), 4 (17 seqs) and 6 (47 seqs), and along with the sequence for our HCV cDNA construct aligned using the ClustalW multiple sequence alignment tool (genotype 1b) (Fig. 3.13). We found that within genotype 1a and 1b of HCV, the four amino acids responsible for mediating elongin C binding are highly conserved, both as to amino acid residue and spacing between them; the initial valine residue is followed by two random amino acids before a leucine residue, which is followed by a cysteine residue after three more amino acids and finally, there is a leucine after four more amino acids. These amino acids were not as highly conserved in the other viral genotypes examined.

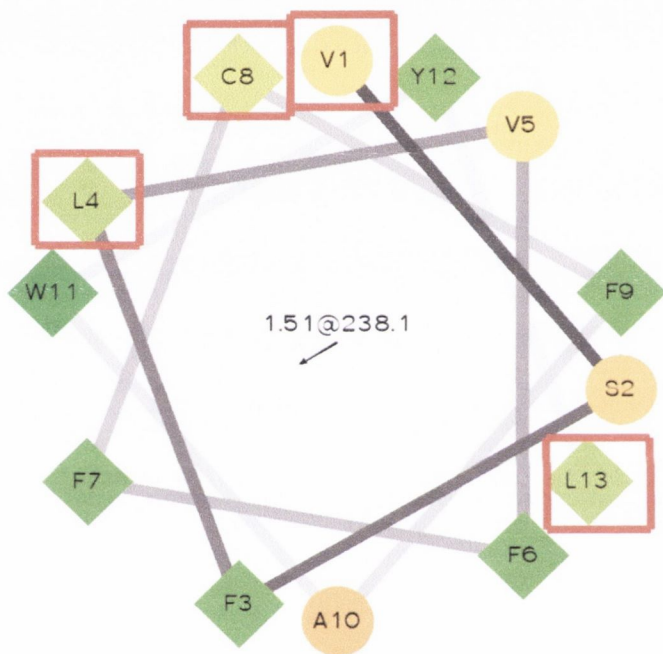
Construct G1b	VSFLVFFCFAWYLK
G1a	VSFLVFFCFAWYLK
G2	WFFIFFVA-AWYLK
G3	GWYLVAFCAAWYVR
G4	WYAIFFICIAWHVK
G6	WWGLLFLCAWYVK
Consensus	V--L---C----V-

Figure 3.13. The Elongin C binding site in different HCV genotypes

Consensus sequences were generated for different HCV genotypes using the consensus maker tool in the Los Alamos HCV sequence database for genotype 1a (325 seqs), 2 (30 seqs), 3 (10 seqs), 4 (17 seqs) and 6 (47 seqs). The elongin C binding site in p7 for each consensus sequence was aligned using ClustalW multiple alignment tool. Identical amino acids are in yellow and similar amino acids are in grey.

3.2.10 Orientation of the elongin C binding site in p7

The putative elongin C binding site in HCV is in a region of p7 that is known to form an α -helix [183], therefore any residues that can theoretically interact with a protein should be orientated on the same side of the helix. To examine this, a helical wheel was generated using an online helical wheel projection programme (Fig. 3.14). The first three residues known to mediate elongin C binding were found to be all facing the same direction of the α -helix.



Elongin C binding site p7: **V**S**F****L**V**F**F**C**FAW**L**

Figure 3.14. Helical wheel projection of the elongin C binding site

A helical wheel was generated using an online helical wheel generator tool (<http://rzlab.ucr.edu/scripts/wheel/wheel.cgi>) to visualise the orientation of the residues of the elongin C binding site in p7 of HCV. Circles are hydrophilic residues, diamonds are hydrophobic residues. Extent of green colour correlates with extent of hydrophobicity. Residues with no hydrophobicity are coloured yellow.

3.2.11 The E2 protein of HCV has a cul-2 binding site

All E3 ligases that bind to an elongin C protein must also bind to a cul-2 or cul-5 protein to mediate targeting of proteins for proteasomal degradation by attachment of poly-ubiquitin chains [29]. The programme FuzzPro from the EMBOSS suite of bioinformatic software (www.emboss.org) was used to search our HCV construct for the presence of either cul-2 or cul-5 binding sites, based on motifs used by other E3 ligases known to bind these proteins. This search identified a possible cul-2 binding motif within the E2 protein of HCV. ClustalW multiple sequence alignment tool was then used to align this putative binding site with known cul-2 binding proteins and the possible binding site within genotype 1a of HCV (Fig. 3.15). The cul-2 binding site in HCV E2 contains amino acids that are conserved and have similar spacing to amino acids known to mediate cul-2 binding in other proteins.

RSV NS1	SKRLSDSVMT-DYMNQISDLL
VHL	SLVKPENYRRLDIVRSLYEDL
Construct (g1b)	SSIASWAIK-WETVVLLFLLL
HCV g1a	SSIASWAIK-WETVVLLFLLL
Consensus	S---S--I---Y---L--LL

Figure 3.15. The cul-2 binding site of HCV

ClustalW was used to align the cul-2 binding proteins NS1 (from RSV) and VHL tumour suppressor protein with our HCV construct (genotype 1b) and HCV genotype 1a.

3.2.12 The cul-2 binding site is conserved in genotype 1 of HCV

Consensus sequences for different HCV genotypes were generated by retrieving sequences of the E2 protein from known genotypes taken from the Los Alamos HCV Sequence Database. Consensus sequences were generated using the consensus maker tool for genotype 1a (245 seqs), 2 (33 seqs), 3 (10 seqs), 4 (21 seqs) and 6 (47 seqs) and aligned using the ClustalW multiple sequence alignment tool (Fig. 3.16). ClustalW multiple sequence alignment of consensus sequences for each genotype revealed that the four amino acids responsible for mediating elongin C binding are highly conserved in genotype 1a and 1b of HCV, but not in other genotypes examined.

Construct G1b	SSIASWAIKWEYVVLLFLLL
1A	SSIASWAIKWEYVVLLFLLL
2	PALTKYIVKWEYVVLLFLLL
3	SGMBGWALKWEFVILVFLLL
4	SAVVSWALKWEYV-LAFLLL
6	SSVVSWAVKWEYVVLAFLVL
Consensus	S---S--I---Y---L--LL

Figure 3.16. The cul-2 binding site in different HCV genotypes

Consensus sequences were generated for various HCV genotypes using the consensus maker tool in the Los Alamos HCV sequence database for genotype 1a (325 seqs), 2 (30 seqs), 3 (10 seqs), 4 (17 seqs) and 6 (47 seqs). The cul-2 binding site in E2 for each consensus sequence was aligned using the ClustalW multiple sequence alignment tool.

3.2.13 Orientation of the cul-2 binding site

The putative cul-2 binding site in HCV is in a region of E2 that is known to form an α -helix [183], therefore residues could be involved in protein interaction should have the same orientation on the helix. To examine this, a helical wheel was generated and analysed (Fig. 3.17). The majority of residues identified as being critical for cul-2 binding are all orientated to the same side of the α -helix.

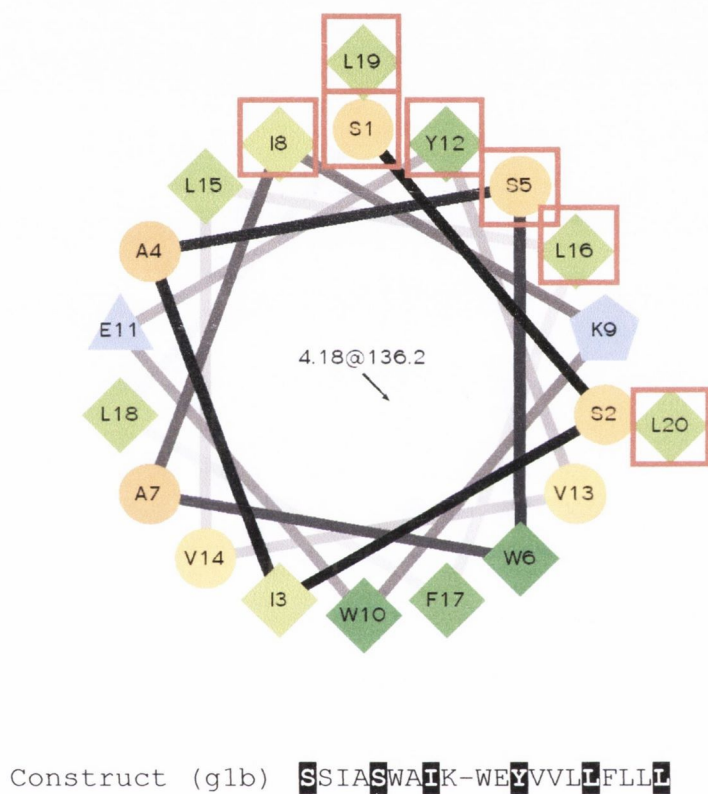


Figure 3.17. Helical wheel projection of the cul-2 binding site

A helical wheel was generated using an online helical wheel generator tool (<http://rzlab.ucr.edu/scripts/wheel/wheel.cgi>) to visualise the orientation of the cul-2 binding site in E2 of HCV. Circles are hydrophilic residues, diamonds are hydrophobic residues, triangles are potentially negative charged residues and pentagons are potentially positively charged residues. Extent of green colour correlates with extent of hydrophobicity. Residues with no hydrophobicity are coloured yellow. Residues with a potential charge are light blue.

3.2.14 HCV NS2 protein has an elongin C binding site

As we has identified an elongin C binding site in p7 and a cul-2 binding site in E2, we further searched the HCV genome for any additional binding sites. Using FuzzPro software from the EMBOSS site (www.emboss.org), we further searched our HCV construct for the presence of additional cul-2 or elongin C binding sites. This led to the identification of a second possible elongin C binding site, within HCV NS2 protein. Multiple sequence alignment of HCV NS2 protein with known elongin C binding proteins demonstrated that the amino acid residues critical for elongin C binding are highly similar and correctly positioned within this motif (Fig. 3.18).

RSV NS1	VALLKITFYTDKLT
HIV vif	VGSLQYLALAAL-I
VHL	VYTLKEPCLQ--VV
Construct (glb)	VQGLLRICALARKI
HCV gla	VQGLLRICALARKM
Consensus	V--L---C-----I/V

Figure 3.18. The elongin C binding site in NS2

ClustalW sequence alignments of elongin C binding sites in other proteins (NS1 protein of RSV, HIV vif protein and VHL tumour suppressor protein) was used to identify an elongin C binding site in the NS2 protein of HCV, both in our genotype 1b HCV construct and HCV genotype 1a. Conserved consensus amino acids are highlighted.

3.2.15 The elongin C binding site in NS2 is highly conserved in genotype 1

As we found that the elongin C binding site in p7 and the cul-2 binding site in E2 were highly conserved in genotype 1 of HCV, we sought to determine whether the elongin C binding site in NS2 was also conserved. Consensus sequences were generated using the consensus maker tool in the Los Alamos HCV Sequence Database for genotype 1a (245 seqs), 2 (33 seqs), 3 (10 seqs), 4 (21 seqs) and 6 (47 seqs) and aligned using the ClustalW multiple sequence alignment tool (Fig. 3.19). As with the other binding sites, the elongin C binding site in NS2 was highly conserved in genotype 1, but not in the other, more IFN- α sensitive, genotypes.

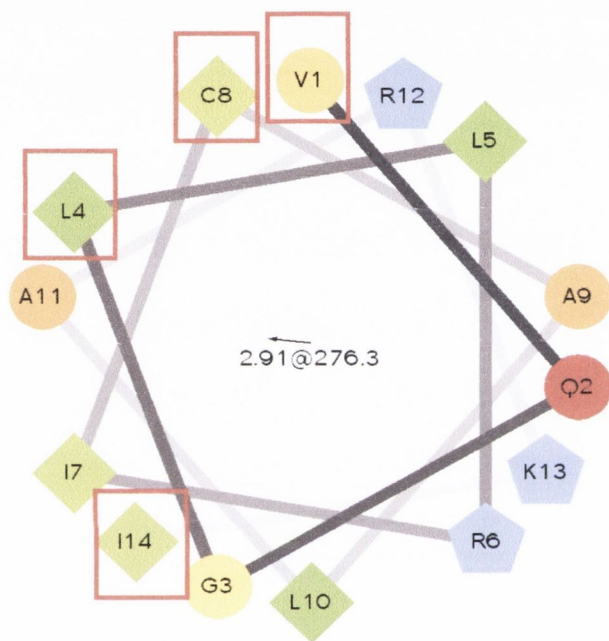
Construct G1b	VQGLLRICALARKI
1A	VQGLLRICALARKM
2	AHALLRVCTLVRHL
3	AHVLVRLCMLVRSV
4	AHALIKICSLLRGV
6	AHILLRACLLVR--
Consensus	V--L---C-----I

Figure 3.19. The elongin C binding site is conserved in genotype 1 of HCV

Consensus sequences were generated for various HCV genotypes using the consensus maker tool in the Los Alamos HCV sequence database for genotype 1a (325 seqs), 2 (30 seqs), 3 (10 seqs), 4 (17 seqs) and 6 (47 seqs). The elongin C binding site in NS2 for each consensus sequence was aligned using the ClustalW multiple sequence alignment tool.

3.2.16 Orientation of the elongin C binding site in NS2

The region of NS2 containing the newly identified elongin C binding site is in an α -helix. Therefore for these conserved amino acids to interact with a protein, they must be facing the same direction in the α -helix. A helical wheel was generated for the elongin C binding site and the first three of the conserved amino acids were in the same orientation (Fig. 3.20).



Elongin C binding site NS2: **V**Q**G**LLR**I****C**ALARK**I**

Figure 3.20. The orientation of the elongin C binding site in NS2

A helical wheel was generated using an online helical wheel generator tool (<http://rzlab.ucr.edu/scripts/wheel/wheel.cgi>) to visualise the orientation of the cul-2 binding site in E2 of HCV. Circles are hydrophilic residues, diamonds are hydrophobic residues and pentagons are potentially positively charged residues. Extent of green colour correlates with extent of hydrophobicity. Residues with no hydrophobicity are coloured yellow. Extent of red colour correlates with extent of hydrophilicity. Residues with a potential charge are light blue.

3.2.17 Cloning of the E2-p7-NS2 region of HCV

Having identified putative elongin C binding sites in p7 and NS2 and a cul-2 binding site in E2, we assessed whether these HCV proteins could inhibit IFN- α signalling. HCV is transcribed as a polypeptide, with E2, p7 and NS2 in linear alignment. By introducing restriction sites for Hind III before E2 and Xho I after NS2 in our HCV cDNA construct, this region was cloned into the vector pcDNA3.1. Expression of E2NS2 was confirmed by visualising NS2 expression using immunoblotting (Fig. 3.21).

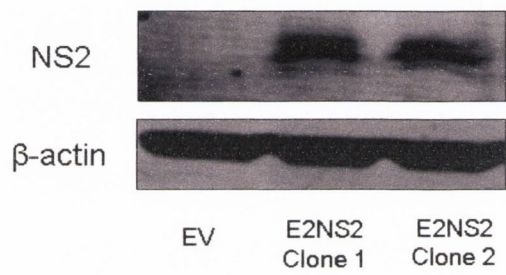


Figure 3.21. Expression of E2NS2 in Huh-7 hepatocytes

Huh-7 hepatocytes were transfected with either pcDNA3.1 (EV) or 2 different E2-p7-NS2 (E2NS2) clones and probed with NS2 antibody to confirm protein expression. β -actin was used as an internal control.

3.2.18 E2NS2 reduces STAT3 expression

Initially, we wanted to determine whether expression of E2-p7-NS2 (E2NS2) could affect the expression of STAT3, as full length HCV had. Huh-7 hepatocytes were transfected with EV, E2NS2 or HCV. Expression of either E2NS2 or HCV resulted in reduced STAT3 protein expression compared to cells transfected with EV (Fig. 3.22).

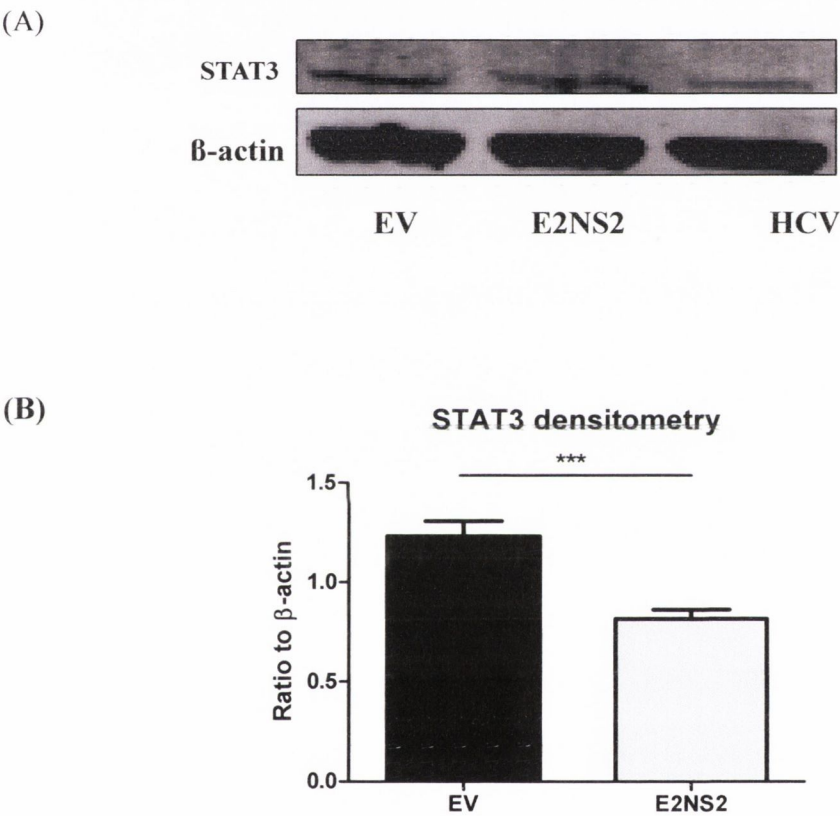


Figure 3.22. E2NS2 inhibits expression of STAT3

(A) Huh-7s were transfected with pcDNA3.1 (EV), E2NS2 or HCV for 16h and STAT3 protein expression was analysed using western blotting (n=3). β-actin was analysed as a loading control. (B) Huh-7s were transfected with pcDNA3.1 (EV) or E2NS2 for 16h and STAT3 protein expression was analysed using western blotting n=6. β-actin was used as a loading control and densitometry analysis was used to quantify protein levels. A ratio of STAT3 to β-actin protein levels were graphed and Student’s T test was used to analyse statistical differences between the two groups. ***p<0.001.

3.2.19 ENS2 inhibits activation of ISRE and GAS promoter elements

To examine the effect of E2NS2 on IFN- α signalling, we quantified the induction of ISRE and GAS luciferase in the presence of E2NS2, or EV and HCV as positive and negative controls, respectively. We showed that E2NS2 significantly inhibited both ISRE (Fig. 3.23A) and GAS luciferase (Fig. 3.23B) induction, even more profoundly than with full length HCV. This was evident at all time points of IFN- α stimulation examined.

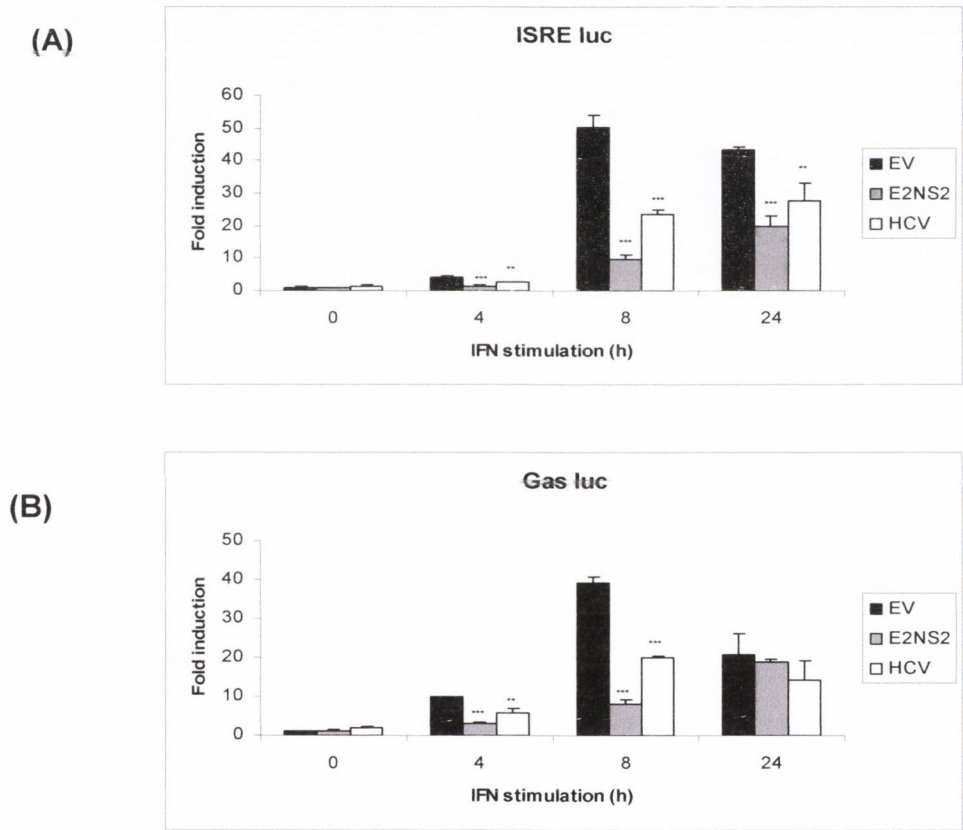


Figure 3.23. E2NS2 is an effective inhibitor of ISRE and GAS luciferase induction

Huh-7 cells were transfected with firefly reporter plasmid, either (A) ISRE-luciferase or (B) GAS-luciferase, TK-Renilla reporter plasmid and pcDNA3.1, E2NS2 or HCV. The cells were stimulated with 1000IU IFN- α for 0, 4, 8 or 24h and then assayed for ISRE or GAS activation. Results are expressed as mean fold induction $\pm$ SD and are representative of at least three experiments, each performed in triplicate. Statistical differences analysed using Student's T test. ** $p < 0.01$ or *** $p < 0.001$ compared to EV.

3.2.20 E2NS2 inhibits ISG induction

To examine the effect of E2NS2 on ISG induction, Huh-7 hepatocytes were transfected with EV or E2NS2 and gene induction of PKR and MxA was assessed. Both PKR and MxA induction was significantly inhibited in the presence of E2NS2 ($p<0.05$) (Fig. 3.24).

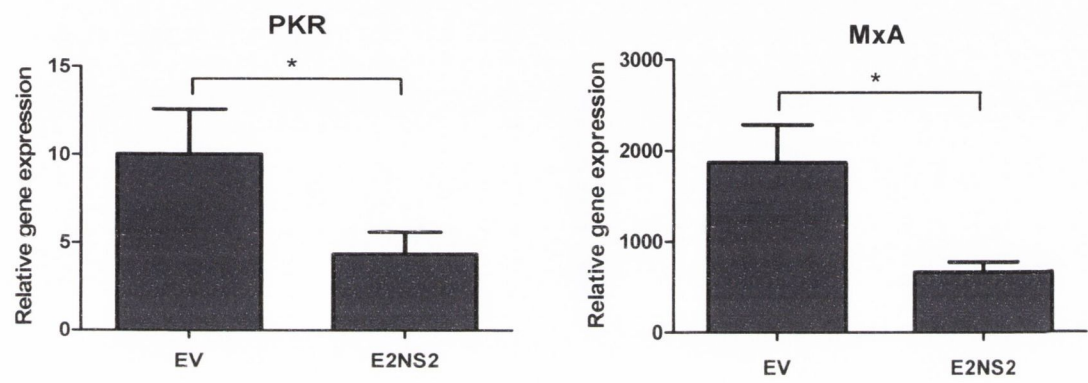


Figure 3.24. E2NS2 inhibits induction of PKR and MxA

Huh-7 hepatocytes were transfected with either pcDNA3.1 (EV) or E2NS2 and stimulated with 1000IU IFN- α for 4h. qRT-PCR was used to quantify expression of PKR and MxA. All samples are normalised to RPS15 housekeeping control. N=4; statistical differences analysed using Student’s T test, $*p<0.05$.

3.2.21 Designing novel peptides against the elongin C binding site in p7

We postulated that if HCV could act as an E3 ligase by recruiting elongin C to block IFN- α signalling, disrupting the interaction between HCV and elongin C would restore IFN- α responsiveness. To test this, a peptide mimicking the elongin C binding site in the p7 protein of HCV was synthesised (Table 3.1). This peptide was designed to have a C-terminal tail of 9 arginines to allow the peptide to penetrate the cell without needing to transfect it. A control scrambled peptide was also designed which contained the same amino acids of the elongin C binding site in p7, but these amino acids were in a random scrambled order.

	Elongin C (p7)
Peptide	9R-VSFLVFFCFAWYL
Scrambled	9R-WVFLFFFYLACSV

Table 3.1 Peptides designed to block HCV inhibition of IFN- α signalling
Sequences for peptides designed against the elongin C binding site in p7. Each peptide is designed to contain a 9 polyarginine tail at the N-terminus.

3.2.22 An inhibitory peptide against the elongin C binding site in p7 can partially recover STAT3 expression

To investigate if the inhibitory peptide against the elongin C binding site in p7 restored STAT3 expression, we treated HCV transfected Huh-7s with the inhibitor peptide against the elongin C binding site in p7. We found that treatment with this peptide alone slightly recovered STAT3 protein expression (Fig. 3.25).

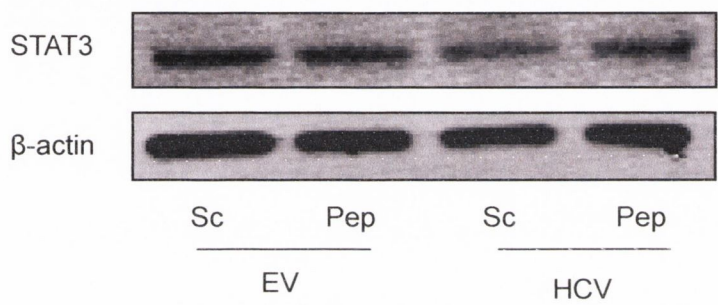


Figure 3.25. Analysis of STAT3 protein expression in the presence of inhibitory peptide

Huh-7s were transfected with 5µg EV/HCV followed by treatment with 5µM of either the scrambled or inhibitory peptide for 16 hours. Cells were lysed and STAT3 was analysed using western blot (n=2). β-actin was used as an internal control.

3.2.23 Designing novel peptides against the cul-2 binding site in E2 and the elongin C binding sites in NS2

A peptide designed against the elongin C binding site in p7 slightly enhanced STAT3 protein expression, therefore we designed similar peptides against the cul-2 binding site in E2 and the elongin C binding site in NS2 (Table 3.2). These were designed to mimic the cul-2 and elongin C binding sites, with the addition of an N-terminal polyarginine tail to facilitate cell entry. Corresponding scrambled peptides were also designed.

	Cul-2 (E2)	Elongin C (NS2)
Peptide	9R-SWAIKWEYVVLLFLLL	9R-VQGLLRIC
Scrambled	9R-YVLFLSELKWLI AVLW	9R-CVIGLQLR

Table 3.2. Peptide sequences for the peptides designed against the cul-2 binding site in E2 and the elongin C binding site in NS2

Peptide sequences for inhibitory peptides designed against the cul-2 binding site in E2 and the elongin C binding site in NS2 with scrambled control peptides. Each peptide is designed to contain a 9 polyarginine tail at the N-terminus.

3.2.24 Treatment with a peptide combination restores STAT3 expression

Treatment with a single peptide against the elongin C binding site in p7 could marginally restore STAT3 protein expression; therefore we wanted to determine whether treatment with a combination of peptides, against the cul-2 binding site in E2 and the elongin C binding sites in p7 and NS2, would fully restore STAT3 expression. Transfection with E2NS2 resulted in decreased STAT3 expression whereas treatment with peptides against the elongin C and cul-2 binding sites fully restored STAT3 protein levels (Fig. 3.26).

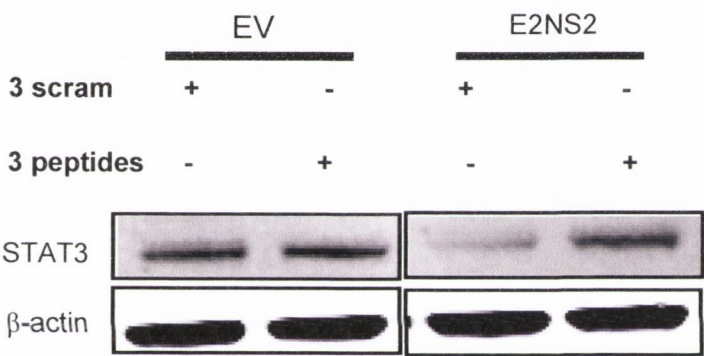


Figure 3.26. Treatment with a combination of inhibitory peptides restores STAT3 protein expression

Huh-7 hepatocytes were transfected with pcDNA3.1 (EV) or E2NS2 for 2h, 3μM of inhibitory peptides against the elongin C binding sites in p7 and NS2, and the cul-2 binding site in E2, were added to the cells for 12h (9μM total peptide concentration). Cells were harvested and STAT3 protein expression was analysed by western blotting (n=3). β-actin was used as a loading control.

3.2.25 Treatment with E3 ligase binding site inhibitor peptides blocks HCV-mediated inhibition of ISG induction

Treatment with all three inhibitory peptides successfully restored STAT3 protein expression in E2NS2 transfected cells. We next aimed to determine whether restoration of STAT3 also rescued ISG induction in these cells. Huh-7 cells were transfected with either EV or E2NS2 and treated with a combination of scrambled control peptides or with the inhibitory peptides. Transfection with E2NS2 and treatment with the scrambled peptides resulted in decreased ISG induction, however treatment with the inhibitory peptides significantly restored both PKR and MxA induction (Fig. 3.27).

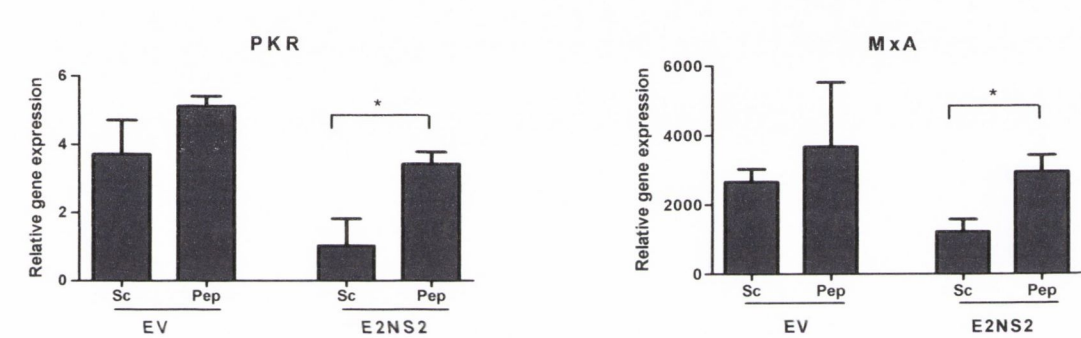


Figure 3.27. Treatment with inhibitory peptides can block HCV inhibition of ISG induction

Huh-7 cells were transfected with either pcDNA3.1 (EV) or E2NS2 for 2h then treated with 3µM peptides of each scrambled/inhibitory peptides (9µM in total), against the elongin C binding site in p7 and NS2 and the cul-2 binding site in E2, for 14h. Cells were stimulated with 1000IU IFN-α for 4h and mRNA was harvested in TRIzol lysis reagent. RT-PCR was used to quantify PKR and MxA expression and samples are relative to untreated control. All samples were normalised to RPS15 internal housekeeping control. N=3; statistical differences analysed using Student’s T test, *p<0.05.

3.3 Discussion

One of the fastest and most effective anti-viral defence mechanisms is the production of the type I IFNs and the activation of the JAK-STAT signalling cascade, which consequently results in the upregulation of 100s of anti-viral genes [99]. Therefore it is not surprising that many viruses have targeted key proteins of this pathway to facilitate viral persistence. Proteasomal degradation of STAT3, a key transcription factor in the regulation of anti-viral gene expression, seems to be a particularly successful immune evasion strategy. Mechanisms that result in STAT protein degradation have been evolved by many viruses such as Mumps virus, which can degrade both STAT1 and STAT3 and RSV, which can degrade STAT2 [29]. Viruses that degrade STAT proteins hijack a natural host mechanism of protein recycling, and by forming their own E3 ligase complexes, can polyubiquitinate STAT proteins which are subsequently degraded by the 26S proteasome.

STAT1 and STAT2 are commonly thought to be involved in anti-viral IFN- α signalling and the induction of type I IFNs [147,148]. Although STAT3 is known to be activated by type I IFNs and has been postulated to be important for anti-viral responses [114], a definitive role in type I IFN signalling remained elusive. Here we show that depletion of STAT3 expression in a hepatocyte cell line, either through shRNA or siRNA, resulted in severely impaired anti-viral ISG induction, similar in extent to what we found when we knocked out STAT1 protein expression. This may explain why HCV targets STAT3 as well as STAT1 for proteasomal degradation [117,184].

Previous bioinformatic analysis revealed that HCV encodes an elongin C binding site in its p7 protein; this is significant as other viral proteins that mediate STAT protein degradation have the potential to bind to and utilise elongin C. This motif contains 4 amino acids deemed crucial for elongin C binding, particularly the first three highlighted residues in figure 6.4, as these have been shown to be critical for elongin C binding to VHL tumour suppressor [166]. Further analysis of the elongin C binding site in the p7 protein of HCV revealed that this motif was highly conserved in genotype 1 of the virus, the genotype most resistant to IFN- α treatment. The binding site motif was not so highly conserved in other genotypes of the virus. p7 has two α -helices [185] and

the identified elongin C binding site is within the second α -helix, therefore amino acids known to be involved in binding should have the same orientation to allow for protein interaction. We confirmed that the first three of the amino acids categorised as being important for binding were all facing the same direction in the α -helix, providing further information consistent with this being a functional binding site. Additional bioinformatic analysis revealed the presence of an additional elongin C binding site in the NS2 protein of HCV. Again, this was highly conserved in genotype 1 of HCV and was in the correct orientation in the α -helix, as determined through helical wheel analysis, for protein binding.

Proteins that bind to elongin C, such as SOCS proteins and VHL tumour suppressor protein, can also bind to cullin proteins. Therefore we sought to investigate whether HCV contained a motif for binding a cullin protein. We discovered a cul-2 binding motif containing amino acids identified as being relevant in cul-2 binding in the E2 protein of HCV, one of the structural glycoproteins that form the envelope of HCV. *In silico* analysis revealed that these amino acids were highly conserved in genotype 1 of HCV and not in the other genotypes, as we had observed for the elongin C binding site. Again, similar to the p7 protein of HCV, this part of E2 is known to have an α -helical structure [27]. Therefore we confirmed that the majority of these amino acids were clustered on the same face of the α -helix, physically allowing these amino acids to interact with the same protein.

Usually the elongin C and cul-2 binding sites are encoded on a single protein that functions as the E3 ligase and binds both these proteins; however in HCV these binding sites were present in the p7, NS2 and E2 proteins. Interestingly, it is long known that the junction between E2 and p7 and between p7 and NS2 is incompletely cleaved; leading to the generation of E2-p7 and E2-NS2 proteins within infected cells [186]. Studies showing that the p7 protein was critical for infectivity *in vivo* also postulated that the E2-p7 protein has a critical role in infectivity, although this could not be confirmed *in vivo* and no function has been assigned to this protein [29,187,188]. Therefore the discovery of a putative cul-2 binding motif in E2 and elongin C binding sites in p7 and NS2 provides a possible functional role for the intact E2-p7-NS2 (E2NS2) protein.

Bioinformatic analysis informed our hypothesis that the HCV E2-p7-NS2 could function as an E3 ligase and ubiquitinate STAT1 and STAT3, thus leading to their degradation and inhibited IFN- α signalling. To test this hypothesis, we cloned out the E2NS2 region of HCV into another vector which allowed us to assess the function of these proteins specifically. We found that expression of these proteins was sufficient to degrade STAT3, inhibit activation of the ISRE and GAS promoter elements and block induction of crucial anti-viral genes. This is strong confirmation that it is indeed this region of the virus that is responsible for mediating degradation of STAT proteins and inhibition of IFN- α signalling.

Studies from our group previously showed that proteasomal inhibition using MG132 could restore STAT3 protein expression. As proteasomal degradation of proteins is a homeostatic process used in normal protein recycling, we determined to design a more viral specific means of inhibiting STAT3 degradation. We postulated that small peptides which mimicked the elongin C binding sites in p7 and NS2, and the cul-2 binding site in E2, would competitively inhibit the interaction between HCV and elongin C and cul-2, therefore preventing STAT1 and STAT3 degradation. Initially we tested the effect of one peptide inhibitor, which worked against the elongin C binding site in p7, on STAT3 expression. We found that this peptide marginally increased the expression of STAT3, but was unable fully to restore it. Therefore, we treated Huh-7 cells transfected with the E2NS2 construct with all three peptides, to ensure complete inhibition of HCV recruitment of the E3 ligase complex. We discovered that treatment with all three peptides together in combination reversed the inhibition of STAT3 expression by E2NS2, thus allowing for the recovery of IFN- α induced ISG expression in the presence of E2NS2. These results have significant therapeutic implications as IFN- α therapy has high failure rate, particularly for those infected with genotype 1 of the virus. Restoration of IFN- α responsiveness in these patients would mean that not only could they respond to IFN- α therapy, but it may also allow them to naturally clear the virus through restoration of their own endogenous IFN- α -mediated immune response. Therefore, targeting of these highly conserved binding sites may provide a novel therapeutic strategy for the treatment of HCV.

Chapter 4

Anti-viral gene induction in PBMCs from HCV patients

4.1 Introduction

HCV is a liver specific virus previously thought to replicate exclusively in hepatocytes. The ability of HCV to replicate in other cell types, such as circulating leukocytes, has been the subject of much controversy. However, many studies have demonstrated HCV extrahepatic replication and it is becoming generally accepted that HCV is capable of replicating in other locations including immune cells, albeit at a lower level than observed in hepatocytes [189]. HCV has a positive sense ssRNA genome which is copied during replication into a negative strand intermediate. This negative strand intermediate is the template for replication of positive sense ssRNA, which is the template for further genome replication or translation of viral proteins (Fig. 4.1). Therefore, to distinguish between active replication of HCV within PBMCs or only the presence of the virus, the detection of both positive and negative strands of HCV in a cell type is required [190].

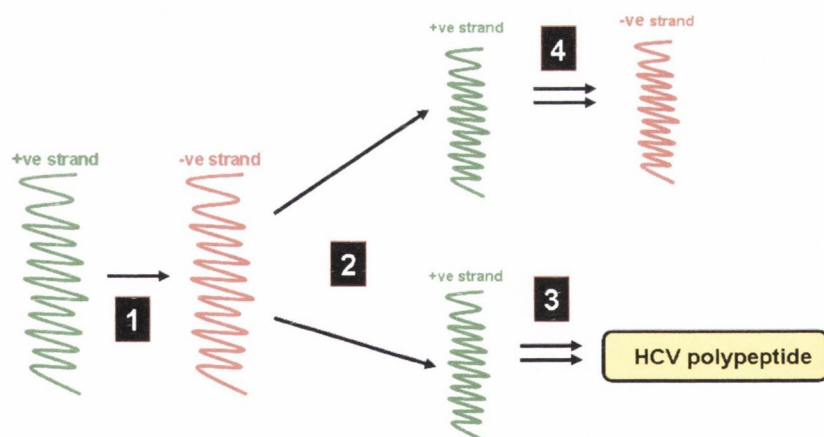


Figure 4.1. HCV replication involves synthesis of negative and positive strands of the viral genome

1. HCV -ve strand replication intermediate is synthesised from the +ve strand viral genome. 2. The -ve strand replication intermediate is transcribed into +ve stranded RNA. 3. This replicated +ve strand RNA can be translated into the HCV polypeptide or 4. The HCV +ve strand RNA can be used as a template to synthesise further -ve stranded RNA.

4.1.1 HCV replication and PBMCs

Experimental and clinical observations suggest that HCV possibly replicates at low levels within peripheral immune cells, thus creating a long lasting viral reservoir. Clinical evidence that HCV exerts effects outside the liver comes from increased incidences of extrahepatic complications in those infected with HCV, including mixed cryoglobulinemia, Non-Hodgkin's lymphoma and glomerulonephritis [29]. Leukocytes such as T and B lymphocytes, monocytes and DCs have all been demonstrated to support HCV replication [191]. Interestingly, the virus found replicating in immune cells may be quite different to the virus found replicating in the liver. In patients chronically infected with HCV, quasispecies of the virus found within the liver differs from the quasispecies detected within DCs of the same individual [192]. The translation efficiency of the quasispecies within the DCs was much lower than within the liver and possibly reflects HCV adaptation to the environment within DCs rather than the hepatocyte intracellular environment. This is consistent with other reports of low level HCV replication in PBMCs compared to hepatocytes and indicates differential compartmentalisation of viral quasispecies within patients. Although viral breakthrough following complete response to IFN- α therapy is rare, its occurrence may be due to the low-level persistence of HCV within PBMCs. For example, some patients who had previously responded to therapy and cleared the virus experienced re-occurrence of HCV after receiving immunosuppressive drugs [193,194]. Given that very low levels of HCV RNA has been detected in PBMCs following apparent viral resolution [195], PBMCs could represent an important viral reservoir capable of causing viral breakthrough. More research is needed to determine the precise cell type within PBMCs that HCV is persisting in and therefore the viral reservoir, as it could have many important implications. For example, if the viral reservoir was B cells, then patients co-infected with HIV would lack the necessary T helper cells to prevent HCV viral breakthrough.

T lymphocytes may play an important role in regulating HCV replication within PBMCs. Depletion of CD8⁺ T cells from PBMCs isolated from HCV patients increases HCV replication in the remainder of the PBMC fraction, as determined by measuring both strands of the replicating virus [196]. CD4⁺ T cells are also likely to regulate

HCV replication within PBMCs as PBMCs cultured *ex vivo* from HCV/HIV co-infected patients, which have reduced CD4+ T cells due to HIV infection, produce more HCV RNA in culture supernatants than patients mono-infected with HCV, indicating increased replication of HCV in PBMCs from co-infected patients due to loss of functional CD4+ T cells [197]. Administration of therapeutic IFN- α strongly upregulates ISGs in PBMCs *in vivo* [198], therefore low level HCV replication within PBMCs could have major implications for response to IFN- α therapy. Indeed, it has been shown that during clinical IFN- α treatment, HCV RNA in PBMCs declines at a slower rate than RNA decline in the serum and those with a faster decline of HCV RNA in their PBMCs had an increased likelihood of viral clearance after treatment [199]. Small amounts of HCV positive and negative strand RNA can still be detected in PBMCs from patients up to 8 years after spontaneously resolving infection or clearing the virus following IFN- α therapy [200,201]. This indicates that PBMCs may be persistently infected with low amounts of viral RNA, creating a latent reservoir of the virus that can be re-activated if the host becomes immunocompromised, such as after liver transplantation [193,202]. We also can detect HCV RNA within PBMCs from HCV infected patients and have shown the expression of the NS2 protein in PBMCs by western blotting (Data provided by Dr. Nigel Stevenson, Fig. 4.2).

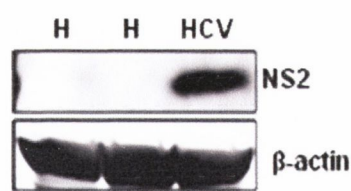


Figure 4.2. HCV NS2 is expressed in PBMCs from HCV infected patients

PBMCs from healthy (H) and HCV infected patients were isolated and lysed in RIPA buffer and ran on an SDS-PAGE gel. NS2 protein expression was analysed. β -actin was analysed as a loading control.

4.1.2 PBMCs from HCV patients and STAT proteins

This evidence suggests that HCV replication within PBMCs has profound effects on the host immune system and response to IFN- α therapy. Therefore, we aimed to investigate IFN- α signalling in PBMCs from HCV infected patients. Our group previously found that expression of STAT1 and STAT3, key transcription factors in the IFN- α signalling cascade, was greatly reduced in the PBMCs from HCV infected patients (Data provided by Dr. Nigel Stevenson, Fig. 4.3).

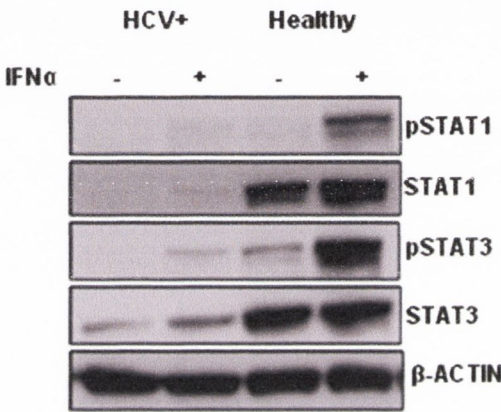


Figure 4.3. STAT1 and STAT3 are absent in the PBMCs of HCV infected patients

Western blot analysis of PBMCs from healthy controls and HCV infected patients, with or without stimulation with IFN- α . Phosphorylation of STAT1 and STAT3 (pSTAT1 and pSTAT3) and total protein levels of STAT1 and STAT3 were analysed. β -actin was analysed as a loading control.

This result was similar to what we had observed in Huh-7 hepatocytes transfected with a HCV DNA construct (Fig. 3.2). Although STAT1 had previously been shown to be absent from HCV cell culture systems [166], this was the first time it was shown to be absent from patient PBMCs. The finding that STAT3 was also absent indicates a profound effect of HCV on the JAK-STAT pathway in circulating leukocytes during HCV infection. STAT3 degradation in PBMCs could be reversed upon proteasomal inhibition (Fig. 3.1), indicating that HCV mediates STAT3 degradation via the proteasome in PBMCs, similar to what we found in Huh-7 hepatocytes. As STAT3 was absent from PBMCs of HCV patients, and we previously showed that STAT3 was

essential in ISG induction in a hepatocyte cell line (Fig. 3.10); we wanted to determine the role of STAT3 in IFN- α induced anti-viral immune responses in PBMCs.

4.1.3 PBMCs from HCV patients and induction of ISGs

PBMCs include major leukocyte populations such as monocytes, DCs, NK cells, T and B lymphocytes. *In vitro* stimulation of T cells, NK cells and monocytes with IFN- α upregulates ISGs and this *ex vivo* upregulation is comparable to *in vivo* stimulation with IFN- α [166]. Therapeutic administration of IFN- α induces anti-viral gene expression in PBMCs from both humans and chimpanzees, with higher ISG upregulation in PBMCs than the liver *in vivo* [203]. As STAT1 and STAT3 are absent in PBMCs from HCV patients, and both are important in IFN- α signal transduction, we sought to examine ISG expression in PBMCs from HCV patients

4.1.4 Viral genotype and ISG induction in PBMCs

Another important issue we aimed to resolve was whether different genotypes of HCV influenced IFN- α induced gene expression in PBMCs. Replicating HCV has been found in PBMCs from patients infected with various viral genotypes [204] and viral genotype is the strongest predictor of response to IFN- α therapy. Less than 50% of those infected with genotype 1 of HCV respond to therapy, whereas around 80% of those infected with genotype 2 or 3 will respond [2]. Viral genotype has also been shown to influence ISG expression within the liver of infected patients; genotype 1 infected patients have higher hepatic ISG expression than those infected with other genotypes and high hepatic ISG expression is linked to non-response to IFN- α treatment [205]. Therefore we aimed to expand our study to determine whether viral genotype could influence ISG expression in PBMCs from HCV patients.

4.1.5 PBMCs and response to IFN- α treatment

Therapeutic administration of IFN- α strongly upregulates ISG expression in PBMCs [198]. However, in HCV infected individuals, STAT1 and STAT3 are absent in patient PBMCs (Fig 4.1). We showed using bioinformatic analysis that genotype 1 of HCV had highly conserved mechanisms for promoting this degradation of STAT proteins compared to other genotypes (Chapter 3). A possible reason for non-response to IFN- α therapy in HCV patients could be due to the loss of STAT1 and STAT3 protein expression in patient PBMCs and a subsequent failure to induce anti-viral ISG expression. Therefore, we assessed IFN- α responsiveness in the PBMCs from patients who failed to respond to IFN- α therapy. If a patient who did not respond to therapy had low ISG induction *in vitro* in their PBMCs, this could represent an important method of analysing the responsiveness of a HCV patient to therapy and could possibly be used as a predictive assay prior to patients commencing IFN- α therapy.

Initially, we evaluated expression of typical anti-viral genes induced by IFN- α such as PKR, OAS and MxA. However, IFN- α can upregulate expression of 100s of ISGs with many diverse cellular functions, therefore we also expanded our study to analyse other anti-viral genes. One such protein upregulated by IFN- α was the chemokine CXCL10, also known as IFN- γ -induced protein 10 kDa (IP-10). CXCL10 is an inflammatory chemokine associated with recruitment of Th1 lymphocytes which signals through the receptor CXCR3. Elevated levels of CXCL10 are associated with several autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus (SLE) [206]. Furthermore, elevated levels of this chemokine have been linked counter-intuitively to non-response in HCV patients. Patients with lower CXCL10 levels pre-treatment were more likely to have sustained virological response than those with high pre-treatment serum levels [207].

Another protein induced by IFN- α , also with a strong link to autoimmune disease, is tripartite motif-containing protein 21 (Trim 21), also known as Rho52 or SSA. Trim 21 is traditionally thought of as being an autoantigen in diseases such as SLE and Sjögren's syndrome [208]; which are frequent co-morbidities of chronic HCV infection [209]. However, a role for Trim 21 in innate immunity and anti-viral defence is emerging. It has been shown that Trim 21 is an E3 ligase that mediates degradation of

IRF3, IRF7 and also IRF8 [210,211]. As Trim 21 is an IFN-inducible protein, it is thought that this degradation is part of a normal regulatory homeostasis to control IFN production. The E3 ligase activity of Trim 21 has also been demonstrated recently to have important, direct roles against influenza and other non-enveloped viruses [212]. The mechanism for this direct effect involves Trim 21 recognising the non-enveloped virus coated with antibody in the cytoplasm of cells, and this recognition allows Trim 21 to target the virus for proteasome-mediated degradation. With these emerging roles for this E3 ligase in innate anti-viral immunity, we decided to investigate whether there was any significance for this IFN- α induced protein in HCV infection.

4.1.6 Aims

The overall aim of this study was to analyse responsiveness of PBMCs from HCV infected patients to *in vitro* IFN- α stimulation. Specifically, we sought to compare ISG expression in PBMCs from HCV infected and uninfected individuals and to analyse IFN- α responsiveness in PBMCs from patients infected with various genotypes of HCV, as viral genotypes respond differently to IFN- α therapy. We hypothesise that reduced expression of STAT1 and STAT3 in PBMCs from HCV patients would have important consequences for response to clinical IFN- α treatment. Therefore, we also examined the response to IFN- α in PBMCs from patients who failed to respond to clinical IFN- α therapy. Proteasomal inhibition restores STAT3 protein expression in PBMCs from HCV patients (Fig. 3.1), hence we aimed to determine the effect of STAT3 recovery on ISG induction in patient PBMCs and determine if viral targeting of STAT3 in PBMCs was due to an important role for STAT3 in anti-viral immunity within PBMCs, similar to what we had demonstrated in Huh-7 hepatocytes in chapter 3.

4.2 Results

4.2.1 PBMCs upregulate anti-viral gene expression in response to IFN- α

Initially, we confirmed that *in vitro* stimulation of PBMCs with IFN- α could upregulate ISG expression. PBMCs were isolated from healthy controls and qRT-PCR was used to quantify ISG induction. All three anti-viral genes examined, PKR, OAS and MxA, were rapidly induced following IFN- α stimulation (Fig. 4.4). Induction of ISG expression was similar at 2h, 4h and 6h after initial stimulation with inter-individual variance observed for all ISGs at all time points examined.

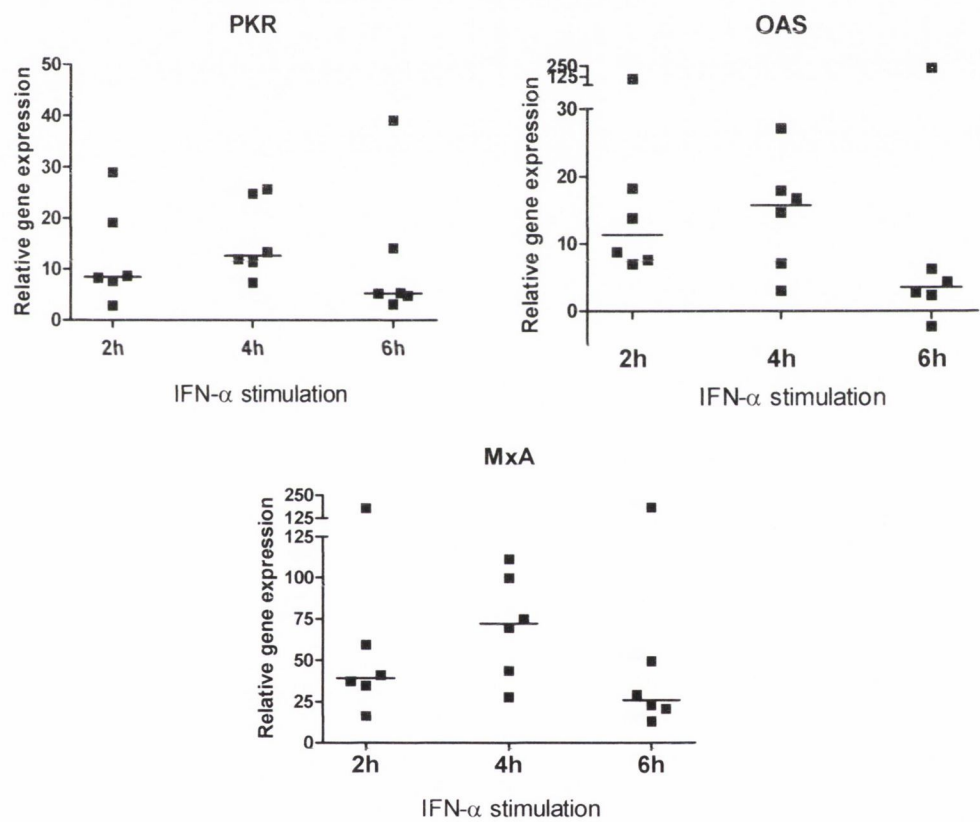


Figure 4.4. qRT-PCR analysis of ISG induction in PBMCs from healthy individuals

2×10^6 PBMCs, isolated from healthy controls ($n=6$), were stimulated *in vitro* for 2, 4 or 6h with 1000IU of IFN- α . PKR, OAS and MxA gene induction was analysed using qRT-PCR. All samples were normalised to endogenous RPS15 expression. Median fold changes are shown, relative to unstimulated controls.

4.2.2 PBMCs from HCV patients upregulate ISGs in response to *in vitro* IFN- α stimulation

To assess whether there was a difference in anti-viral gene induction in the immune cells of healthy and HCV infected patients, we stimulated PBMCs *in vitro* with IFN- α for 2h. IFN- α stimulated induction of PKR, OAS and MxA gene expression was similar in PBMCs from both healthy and HCV infected individuals (Fig. 4.5). Both groups had high diversity in ISG induction, with the extent of induction extremely variable between individuals within either of the groups. Those with high induction of one of the ISGs had similarly high induction of the other two ISGs. None of the ISGs differed significantly in their upregulation between healthy or HCV infected individuals.

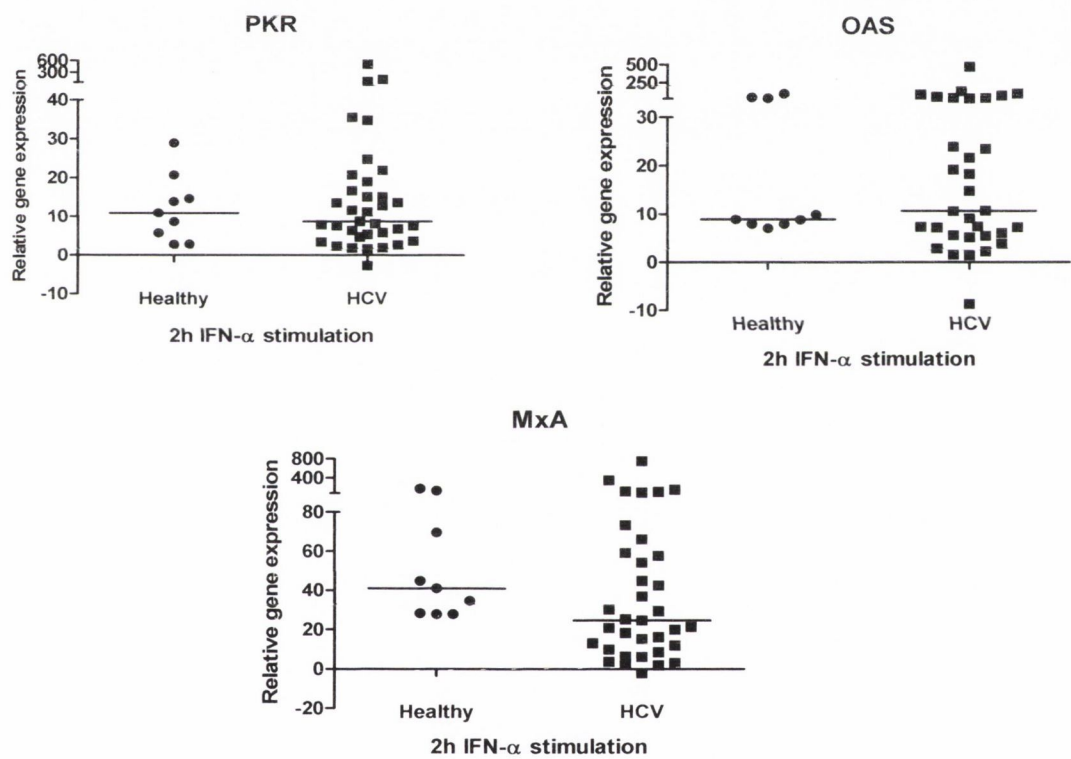


Figure 4.5. qRT-PCR analysis of IFN- α induced ISGs in PBMCs from healthy and HCV +ve patients

2×10^6 PBMCs, isolated from healthy controls (n=9) and HCV patients (n=35), were stimulated with 1000IU IFN- α for 2h. PKR, OAS and MxA gene expression was quantified using qRT-PCR. All samples were normalised to endogenous RPS15 expression. Median fold changes are shown, relative to unstimulated controls.

4.2.3 Endogenous expression of anti-viral genes is similar in healthy controls and HCV infected patients

As ISG induction in both healthy and HCV patient PBMCs was similar following IFN- α stimulation, we examined endogenous levels of ISG expression within PBMCs from those individuals. Endogenous expression of PKR, OAS and MxA was similar in the PBMCs of healthy controls and HCV infected patients (Fig. 4.6). HCV infected patients seemed to exhibit higher diversity in endogenous ISG expression. To test this statistically, the extent in variability in endogenous ISG expression between healthy and HCV infected patients was assessed using a Levene's non-parametric test. The null hypothesis of the non-parametric Levene's test is that both groups have equal variability. Therefore, this test calculates a p-value based on the difference between the extents of variability between 2 groups [213]. The p-values calculated using non-parametric Levene's test were 0.43 for PKR, 0.89 for OAS and 0.38 for MxA. Therefore, variability in endogenous ISG expression is similar in PBMCs from healthy controls and HCV infected patients.

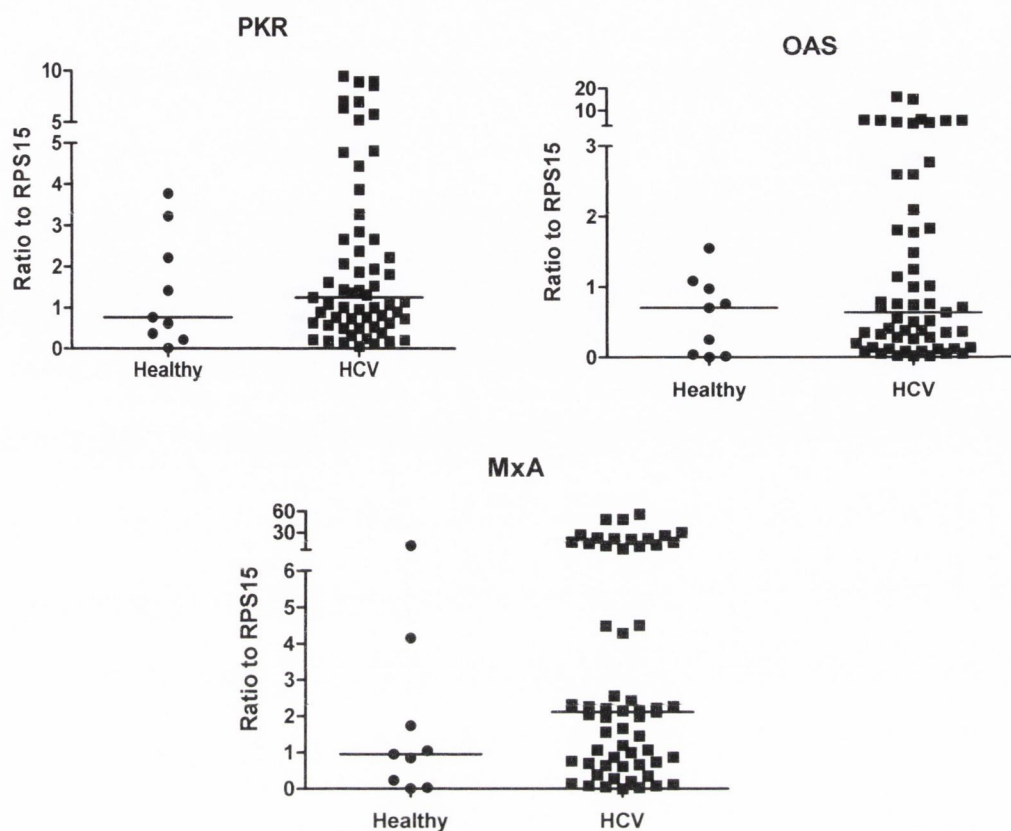


Figure 4.6. qRT-PCR analysis of endogenous ISGs levels in PBMCs from healthy and HCV individuals

PKR, OAS and MxA gene expression was quantified using qRT-PCR from 2×10^6 PBMCs, isolated from healthy controls ($n=9$) and HCV patients ($n=59$). Median fold changes are shown, relative to RPS15 gene expression.

4.2.4 ISG induction is similar in patients infected with genotype 1 and those infected with other genotypes

Patients infected with genotype 1 have very different responses to therapeutic IFN- α than those of other genotypes, therefore we analysed ISG induction in PBMCs in relation to viral genotype. There were no significant differences in induction of PKR, OAS and MxA between healthy controls, genotype non-1 and genotype 1 infected patients (Fig. 4.7). Induction of PKR was similar in healthy controls and patients infected with genotypes other than genotype 1, whereas genotype 1 infected patients appeared to exhibit more diversity in their PKR induction. OAS induction was diverse in healthy and HCV patients, regardless of genotype. MxA induction in the genotype non-1 infected HCV patients showed little variability, whereas healthy controls and patients infected with genotype 1 showed more diverse MxA induction. To assess the extent in variability in ISG induction between genotype 1 and genotype non-1 infected patients, a Levene's non-parametric test was used. When this test was applied to analyse the extent of variability in ISG induction between genotype 1 and genotypes non-1 infected patients, we found that genotype 1 infected patients had more diverse ISG induction variability for PKR ($p=0.06$), OAS ($p=0.06$) and MxA ($p=0.055$) compared to genotype non-1 infected patients, although these p -values did not reach significance ($p<0.05$).

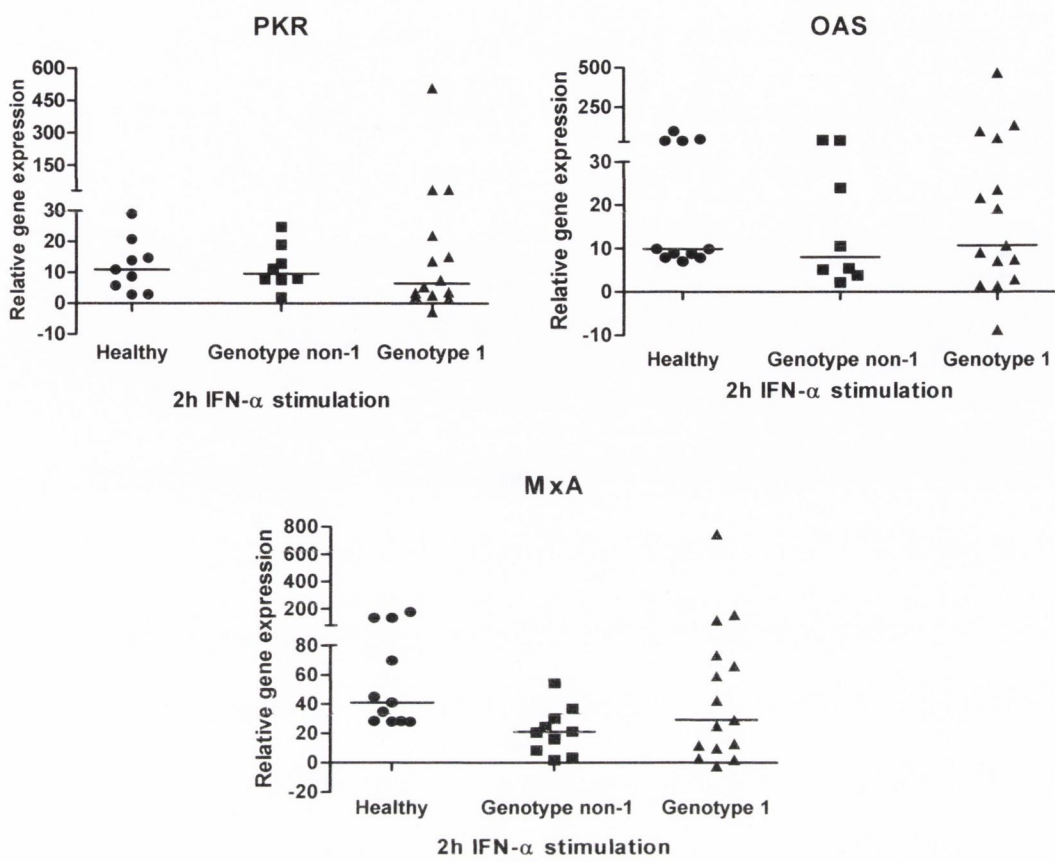


Figure 4.7. ISG induction in PBMCs from HCV patients infected with genotype 1 of the virus and other genotypes

2x10⁶ PBMCs, isolated from healthy controls (n=9), HCV patients infected with genotypes other than genotype 1 (genotype non-1; n=8) and genotype 1 infected patients (n=14), were stimulated with 1000IU IFN-α for 2h. PKR, OAS and MxA gene expression was quantified using qRT-PCR. All samples were normalised to endogenous RPS15 expression. Median fold changes are shown, relative to unstimulated controls.

4.2.5 Genotype 1 infected patients have similar endogenous ISG expression in their PBMCs compared to genotype non-1 infected patients

In the liver, endogenous expression of hepatic ISGs is higher in those infected with genotype 1 than genotypes 2 and 3 [205], therefore we sought to investigate endogenous ISG expression in PBMCs from HCV patients of different genotypes and determine whether patients infected with genotype 1 of HCV had higher ISG expression in their PBMCs compared to those infected with other genotypes (genotype non-1). We observed no significant difference in endogenous ISG expression between the healthy controls, genotype non-1 and genotype 1 infected patients (Fig. 4.8). Healthy controls and HCV patients all showed diverse variability in endogenous ISG expression, regardless of viral genotype.

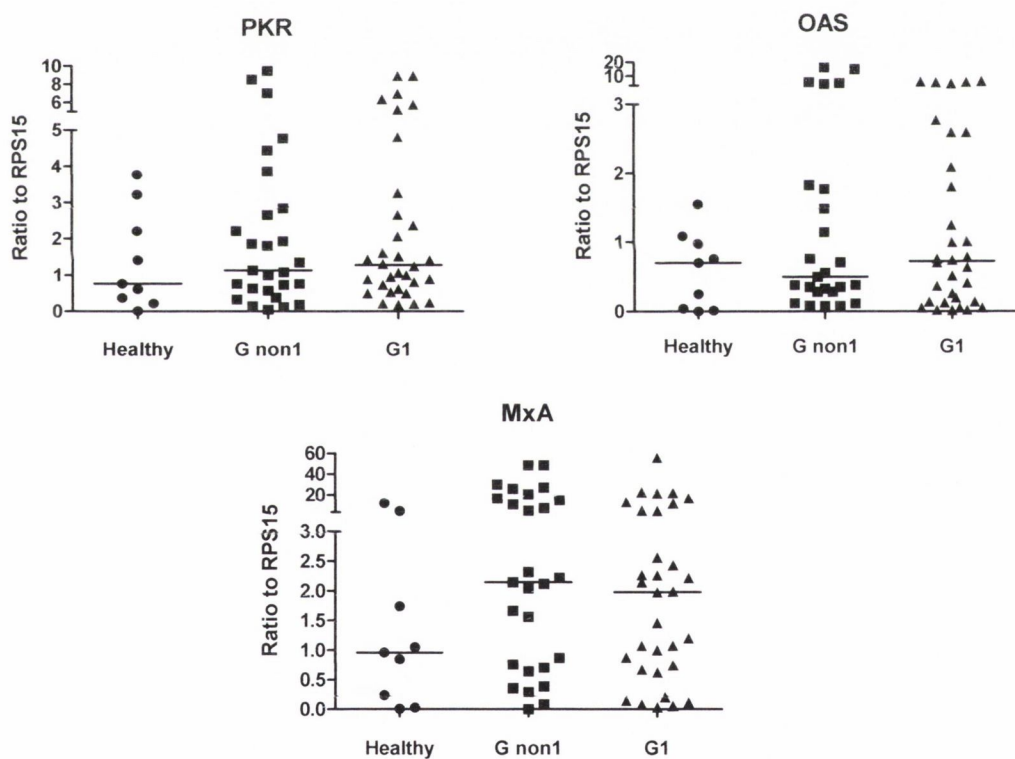


Figure 4.8. Genotype analysis of endogenous ISG levels in PBMCs from HCV infected patients

PKR, OAS and MxA gene expression was quantified using qRT-PCR from 2×10^6 PBMCs, isolated from healthy controls (n=9) and HCV patients infected with genotypes other than genotype 1 (genotype non-1; n=27) and genotype 1 infected patients (n=32). Median fold changes are shown, relative to RPS15 gene expression.

4.2.6 Patients who failed to respond to therapy have low anti-viral gene induction *in vitro*

Unfortunately, many patients infected with HCV fail to respond to clinical IFN- α therapy. Having shown that PBMCs from HCV patients can exhibit a high degree in variability regarding ISG induction *in vitro*, we postulated that perhaps patients who failed to respond to IFN- α therapy (non-responders) would exhibit low ISG induction *in vitro*. These non-responders were patients who were PCR +ve 6 months following IFN- α therapy. As controls we used PBMCs from patients who had previously responded to clinical IFN- α (responders). Responders showed a significantly higher induction of anti-viral genes in response to IFN- α *in vitro* in comparison to non-responder patients (Fig. 4.9). Induction of PKR was 40 fold in responders, whereas in non-responders this induction was only 2 fold ($p < 0.018$). The difference in induction in OAS was even more significant, with responders having a 150 fold induction while non-responders showed a 20 fold mean induction ($p < 0.01$). MxA gene induction in responders was over 200 fold and in non-responders it was less than 10 fold ($p < 0.018$).

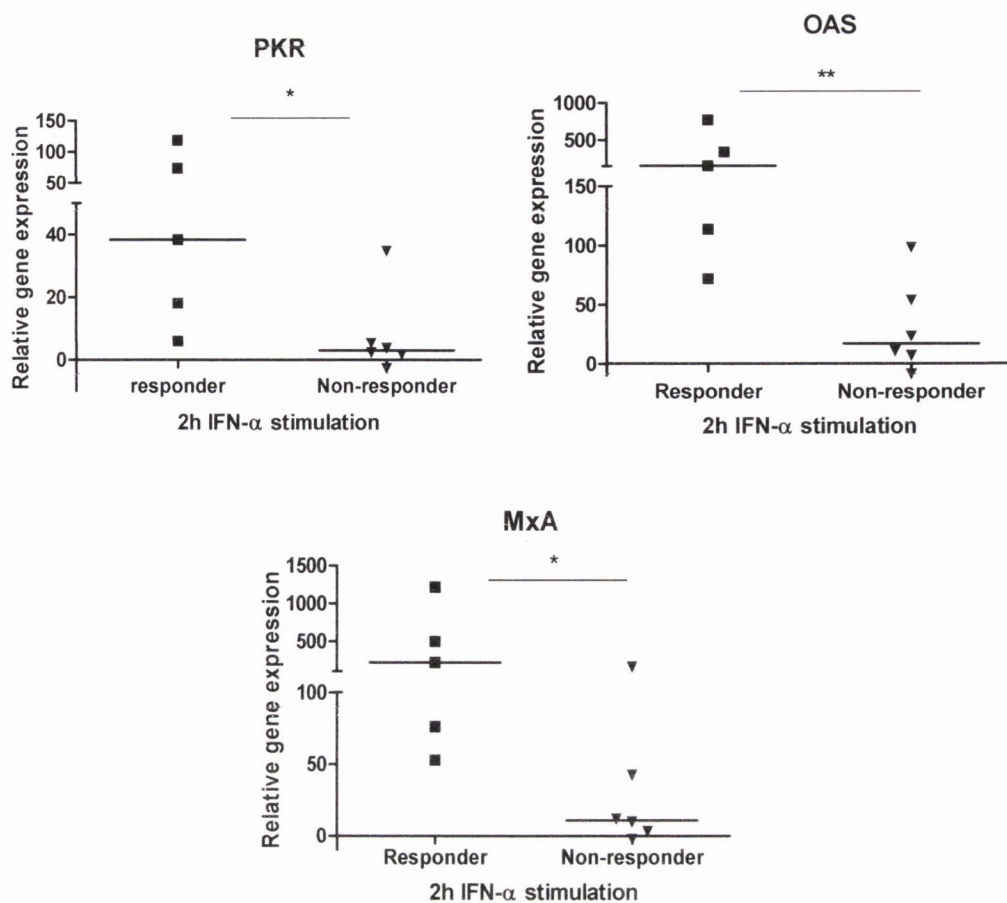


Figure 4.9. ISG induction in PBMCs from HCV +ve patients who failed to respond to therapy

2×10^6 PBMCs, isolated from patients who had completed IFN- α therapy and had responded (responders; $n=5$) or failed to respond (non-responders; $n=6$), were stimulated *in vitro* with 1000IU of IFN- α for 2h. PKR, OAS and MxA mRNA expression was quantified using qRT-PCR. All samples were normalised to endogenous RPS15 expression. Median fold changes are shown, relative to unstimulated controls. Mann-Whitney statistical analysis, * $p<0.05$ and ** $p<0.01$.

4.2.7 Endogenous expression of ISGs is similar in responders and non-responders

HCV patients who fail to respond to IFN- α treatment have higher baseline anti-viral gene expression in their liver prior to IFN- α treatment and this is postulated to contribute to therapy failure due to tolerance to further IFN- α stimulation [212]. We analysed ISG expression in PBMCs from patients who had previously undergone IFN- α therapy and found similar expression of PKR, OAS and MxA in responders and non-responders (Fig. 4.10).

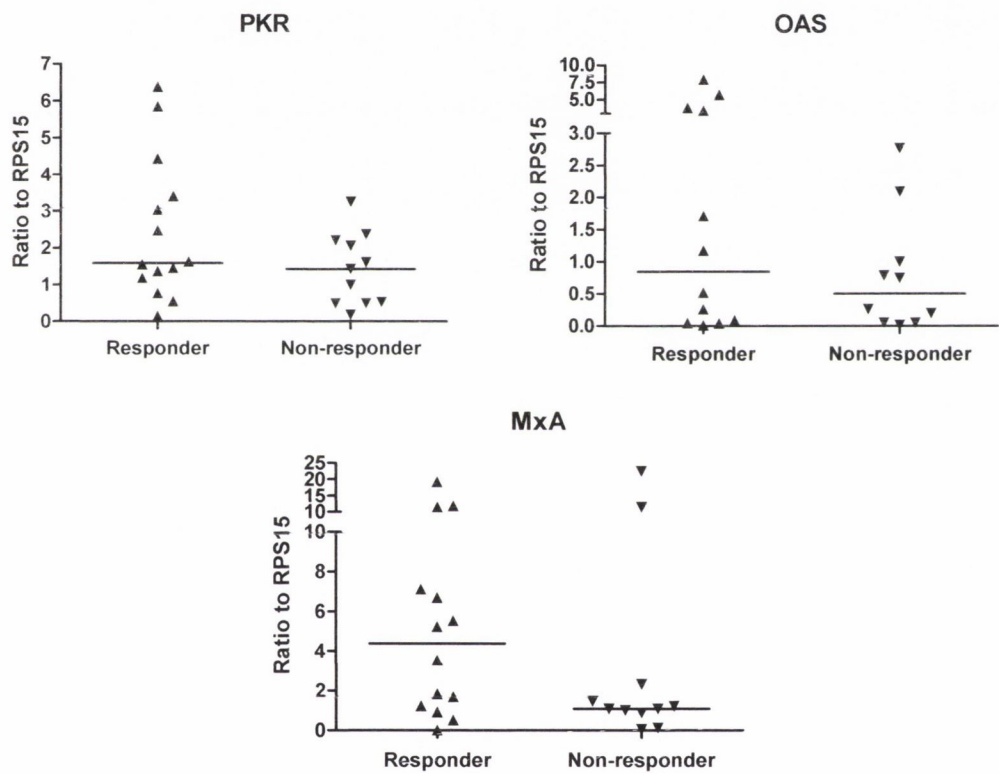


Figure 4.10. Endogenous ISG levels in responders and non-responders to therapy 2×10^6 PBMCs, isolated from patients who had completed IFN- α therapy and had responded (responders; $n=14$) or failed to respond (non-responders; $n=11$), were analysed using qRT-PCR for expression of PKR, OAS and MxA. Median fold changes are shown, relative to RPS15 expression. Mann-Whitney U-tests were used to compare medians.

4.2.8 Difference in induction of ISGs in responders and non-responders is greater in those infected with genotype 1 of HCV

Patients infected with genotype 1 of HCV have the worst response rates to IFN- α therapy [2], therefore analysed ISG expression in PBMCs from patients infected with genotype 1, received IFN- α therapy and either responded or failed to respond. PKR, OAS and MxA gene induction in response to *in vitro* IFN- α stimulation was significantly greater in those who responded to treatment (Fig. 4.11).

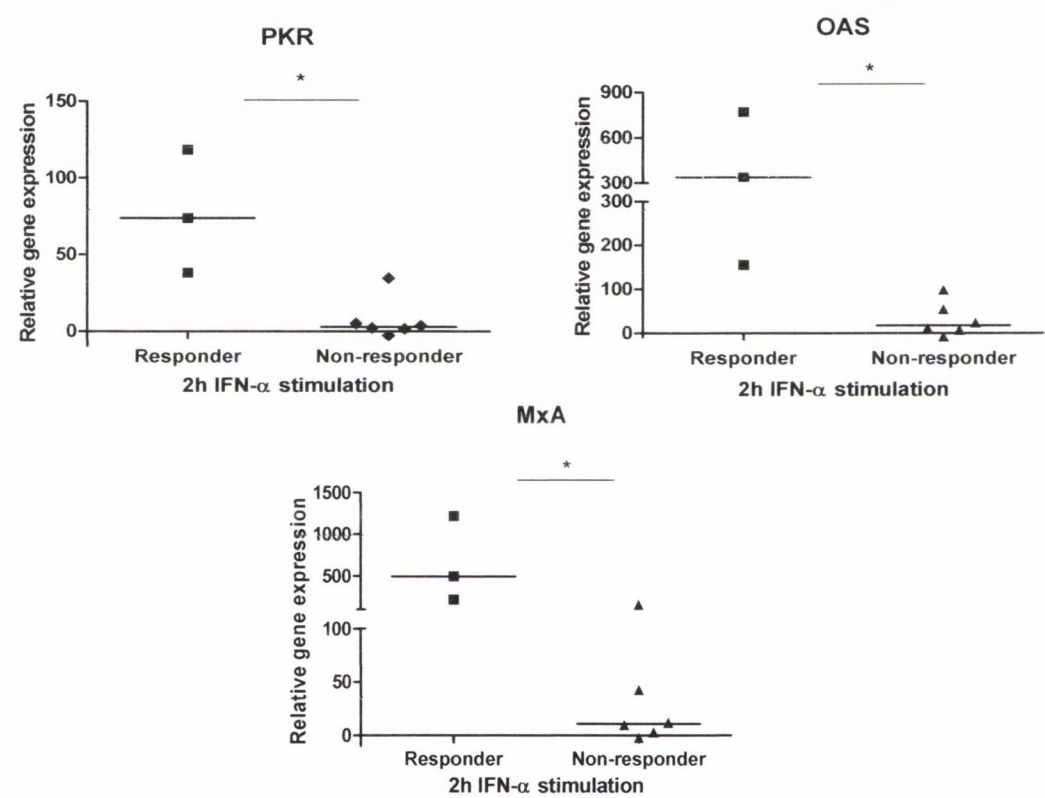


Figure 4.11. ISG induction in genotype 1 infected patients who failed to respond to therapy

2×10^6 PBMCs, isolated from patients who had completed IFN- α therapy and had responded (responders; $n=3$) or failed to respond (non-responders; $n=6$), were stimulated *in vitro* with 1000IU of IFN- α for 2h. PKR, OAS and MxA mRNA expression was quantified using qRT-PCR. All samples were normalised to endogenous RPS15 expression. Median fold changes are shown, relative to unstimulated controls. Mann-Whitney statistical analysis, * $p<0.05$.

4.2.9 IFN- α fails to activate STAT proteins in PBMCs from non-responder patients

We previously found that PBMCs from HCV patients lacked expression of STAT1 and STAT3 proteins (Fig. 4.2). As these transcription factors are crucial for IFN- α signal transduction, and we found that non-responders to therapy had low ISG induction *in vitro* (Fig. 4.11), we assessed STAT1 and STAT3 protein in the PBMCs of HCV patients who failed to respond to therapy. We used flow cytometry to analyse the activation of STAT1 or STAT3 in PBMCs from healthy controls, patients who previously responded to IFN- α therapy and patients who failed to respond. After 15min IFN- α stimulation, healthy controls showed strong phosphorylation of STAT1 and STAT3 (Fig. 4.12A) as did patients who responded to IFN- α therapy (Fig. 4.12B). However, PBMCs from patients who failed to respond to clinical IFN- α therapy induced lower levels of STAT1 and STAT3 phosphorylation after *in vitro* IFN- α stimulation (Fig. 4.12C).

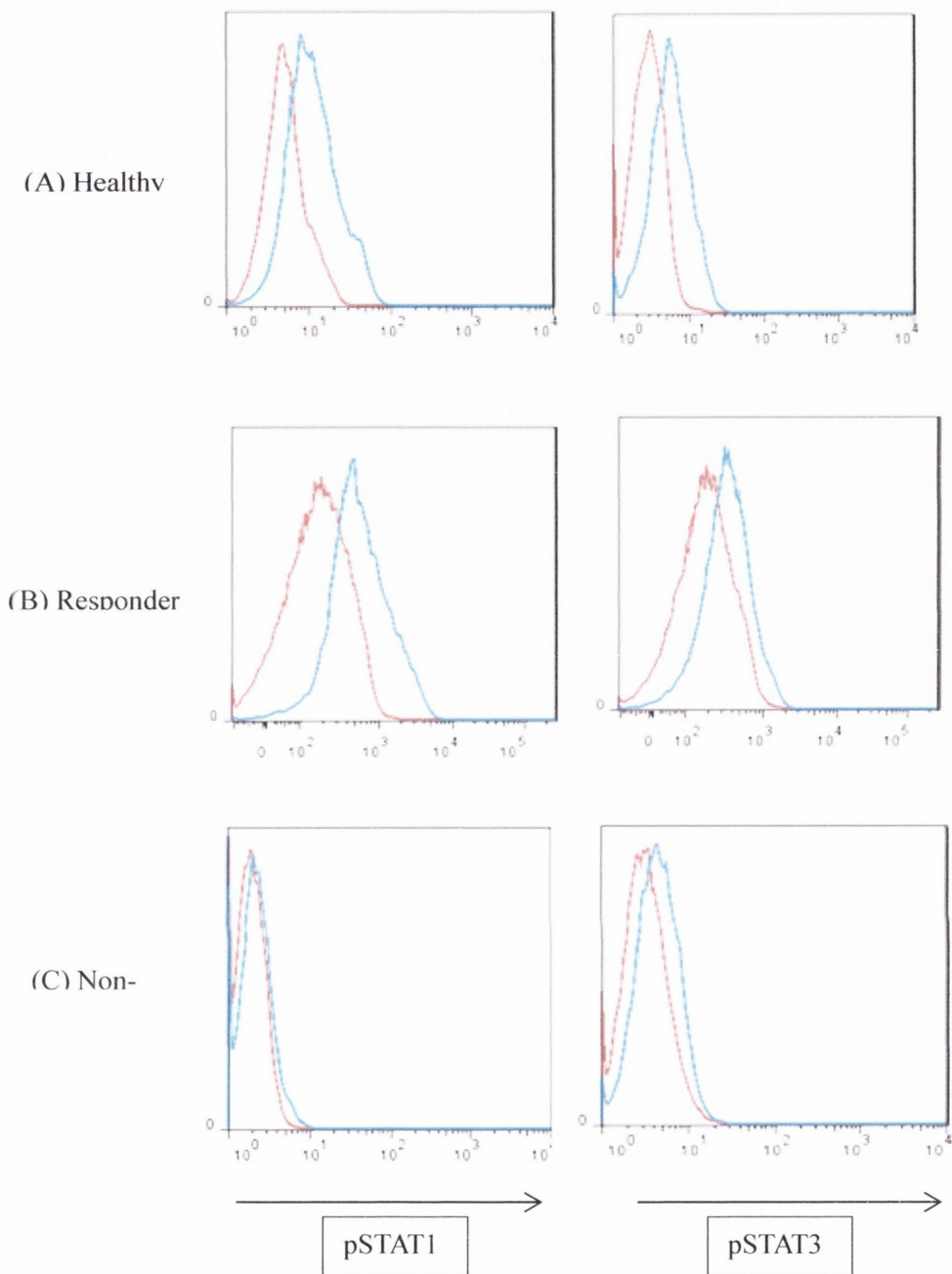


Figure 4.12. Flow cytometric analysis of pSTAT1 and pSTAT3 expression in response to IFN- α

2×10^6 PBMCs, isolated from healthy controls (A; $n=3$), patients who had responded to therapy (B; $n=2$) and HCV patients who had failed to respond to therapy (C; $n=2$), were stimulated *in vitro* with 1000IU of IFN- α for 15 mins. The red line represents unstimulated control and the blue line represents the shift of either pSTAT1 or pSTAT3 following IFN- α stimulation.

4.2.10 STAT1 and STAT3 mRNA levels are similar between healthy patients

Although we had previously demonstrated that depleted STAT3 expression in PBMCs from HCV patients could be recovered following proteasomal inhibition (Fig. 4.2), indicating that HCV targets STAT3 protein rather than mRNA expression, we sought to further confirm that HCV did not affect STAT1 and STAT3 mRNA expression in PBMCs. Therefore, mRNA levels for these proteins were analysed using qRT-PCR in the PBMCs from healthy and HCV infected patients (Fig. 4.13). Endogenous mRNA expression levels of both STAT1 and STAT3 were comparable between healthy controls and HCV infected patients.

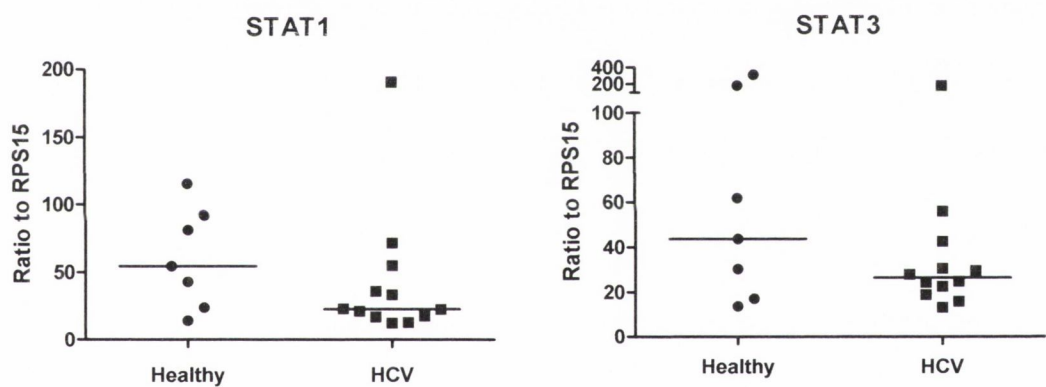


Figure 4.13. STAT1 and STAT3 mRNA expression is similar in PBMCs from healthy controls and HCV infected patients

2×10^6 PBMCs, isolated from healthy controls ($n=7$) or HCV infected patients ($n=12$), were analysed using qRT-PCR for endogenous STAT1 and STAT3 mRNA expression. All samples were normalised to endogenous RPS15 expression. Median fold changes are shown, relative to RPS15 gene expression.

4.2.11 CXCL10 mRNA is elevated in patients who failed to respond to therapy

CXCL10 is another IFN- α upregulated protein that has been associated with non-response to IFN- α therapy. Therefore, we measured mRNA expression for this protein in the PBMCs from non-responder patients. CXCL10 mRNA expression was measured in patients who had responded to clinical IFN- α therapy and patients who failed to respond (Fig. 4.14). Endogenous CXCL10 mRNA was elevated in therapeutic non-responders in comparison to responders ($p<0.03$). There was no significant difference in CXCL10 induction when responders were compared to non-responders, after stimulation with IFN- α .

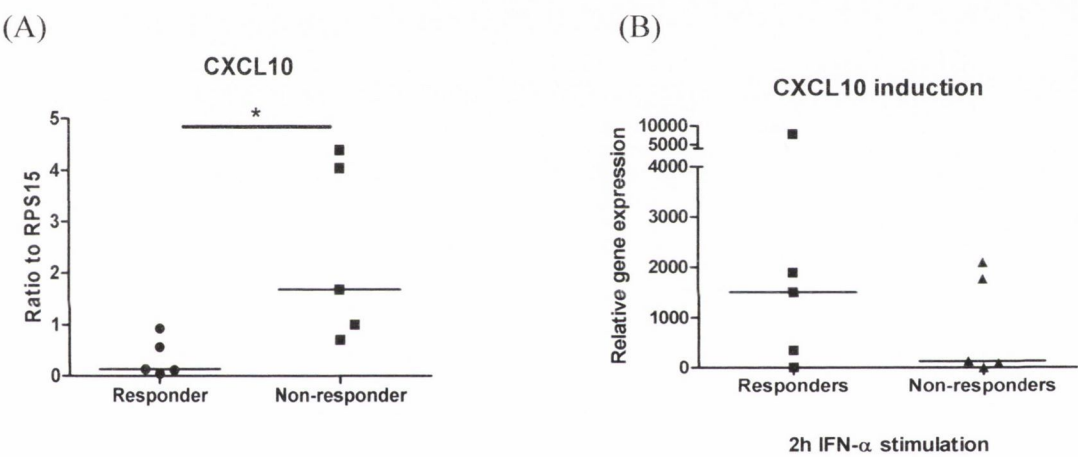


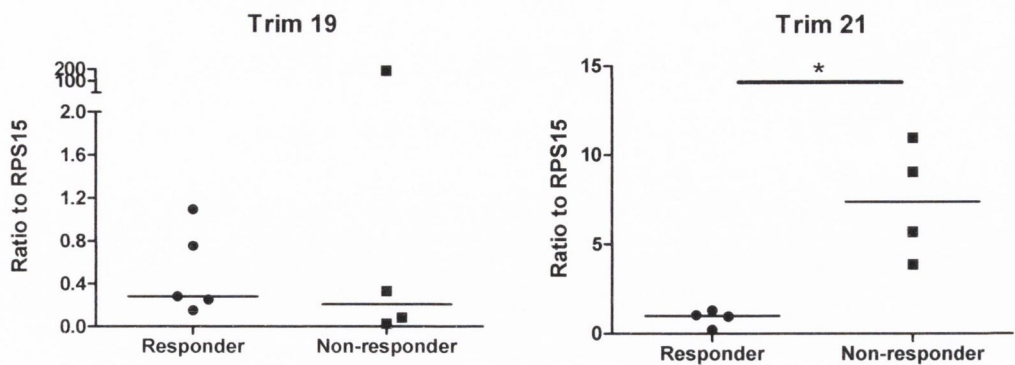
Figure 4.14. Endogenous CXCL10 and CXCL10 induction in PBMCs from responders and non-responders analysed by qRT-PCR

PBMCs were isolated from patients who had previously responded to clinical IFN- α therapy (responders, $n=5$) and from patients who had failed to respond (non-responders, $n=5$) and mRNA was quantified using qRT-PCR. (A) Endogenous CXCL10 expression was measured in 2×10^6 PBMCs, samples relative to RPS15 gene expression (B) CXCL10 was measured following stimulation of 2×10^6 PBMCs with 1000IU of IFN- α , samples normalised to the internal control RPS15 and relative to unstimulated. Median fold changes are shown. Mann-Whitney statistical analysis, $*p<0.05$.

4.2.12 Endogenous mRNA expression levels of Trim 21, but not Trim 19, are elevated in non-responders to therapy

Other proteins with anti-viral roles upregulated by IFN- α are Trim proteins. Endogenous levels of mRNA for both Trim 21 and Trim 19 were quantified by qRT-PCR in PBMCs from patients who responded to therapy and from non-responders (Fig. 4.15A). Endogenous mRNA levels of Trim 21 were significantly higher in non-responders compared to patients who responded to therapy. Contrary to this, there was no significant difference in the endogenous expression levels of Trim 19 between responders and non-responders. Neither was there any difference in the induction of both Trim 21 and Trim 19 following stimulation with IFN- α (Fig. 4.15B). This is in contrast to the difference in ISG induction observed in figure 4.11.

(A) Basal levels



(B) mRNA induction

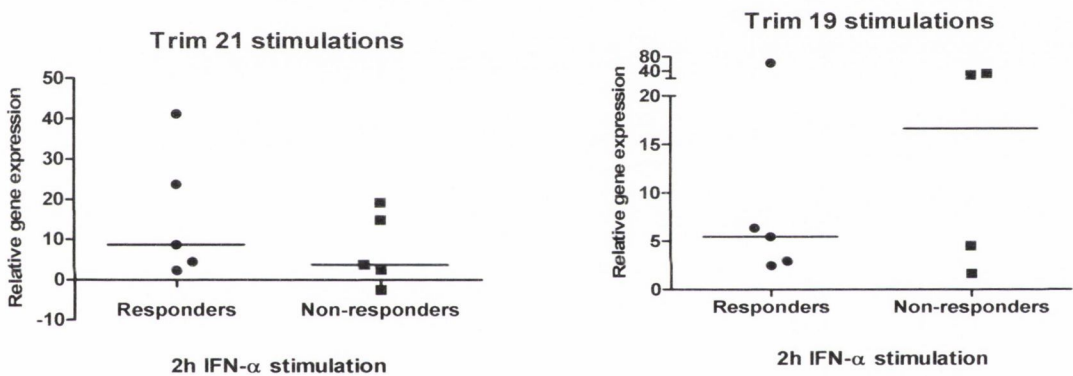


Figure 4.15. Expression of Trim 21 and Trim 19 mRNA in PBMCs from responders and non-responders

PBMCs were isolated from patients who had previously responded to clinical IFN- α therapy (responders, n=5) and from patients who had failed to respond (non-responders, n=5) and qRT-PCR was used to analyse gene expression. (A) Endogenous Trim 21 and Trim 19 mRNA expression levels were measured in 2×10^6 PBMCs. Samples relative to endogenous RPS15 gene expression (B) Trim 21 and Trim19 mRNA expression was measured following stimulation of 2×10^6 PBMCs with 1000IU of IFN- α for 2h. Samples were normalised to the internal control RPS15 and relative to unstimulated. Median fold changes are shown. Mann-Whitney statistical analysis, *p<0.05.

4.2.13 There is no significant difference in IFN- α and IFN- β mRNA between responders and non-responders

We also assessed levels of endogenous type I IFNs in the PBMCs of responders and non-responders, to examine if there was any change in mRNA induction of IFN- α or IFN- β . Endogenous mRNA expression of IFN- α and IFN- β was comparable in responder and non-responder PBMCs (Fig. 4.16). Similarly, there was no difference in the induction of either of these genes in responders and non-responders following stimulation with IFN- α .

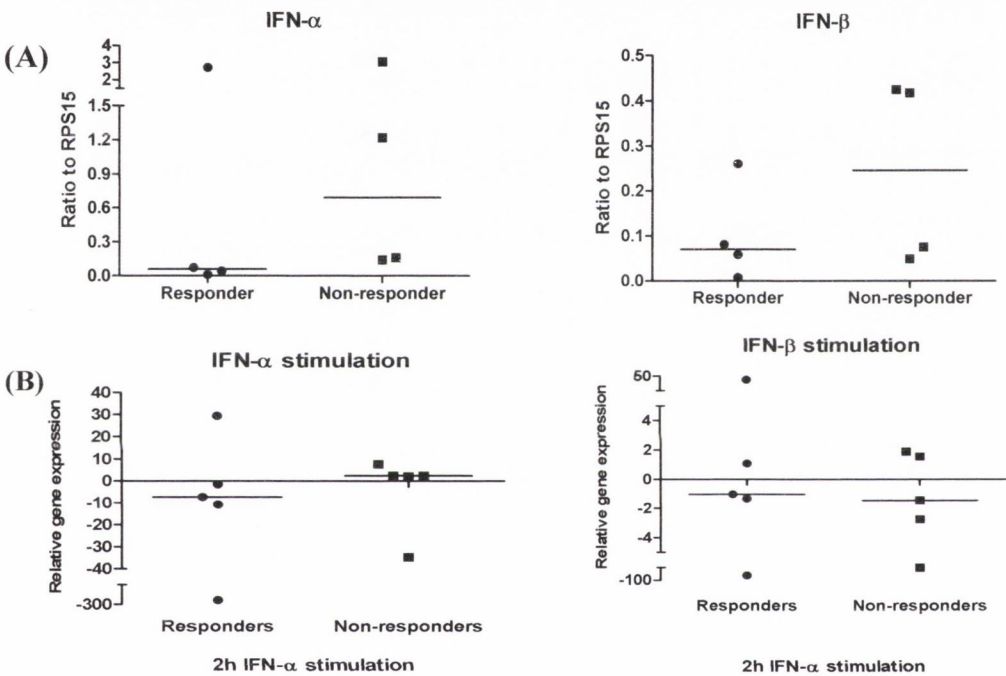


Figure 4.16. IFN- α and IFN- β mRNA expression in PBMCs of non-responders analysed by qRT-PCR

PBMCs were isolated from patients who had previously responded to clinical IFN- α therapy (responders, n=5) and from patients who had failed to respond (non-responders, n=5) and qRT-PCR was used to analyse gene expression. (A) Endogenous IFN- α and IFN- β mRNA expression levels were measured in 2×10^6 PBMCs. Samples were relative to RPS15 gene expression (B) IFN- α and IFN- β mRNA expression was measured following stimulation of 2×10^6 PBMCs with 1000IU of IFN- α . Each sample is normalised to its endogenous RPS15 value and gene expression is relative to unstimulated control. Median fold changes are shown.

4.2.14 Proteasomal inhibition recovers anti-viral gene expression in non-responders

Non-responders to therapy show low anti-viral gene induction in response to IFN- α *in vitro* (Fig. 4.9). As we had previously found that inhibition of the proteasome could restore ISG induction in Huh-7 cells transfected with HCV, we wanted to confirm if ISG induction could be restored in PBMCs from non-responders by blocking the proteasome. PBMCs from non-responders were pre-treated with the proteasomal inhibitor MG132. There was significantly enhanced PKR, OAS and MxA gene expression in these patients following treatment with MG132 and IFN- α compared to treatment with IFN- α alone ($p<0.05$, Fig. 4.17)

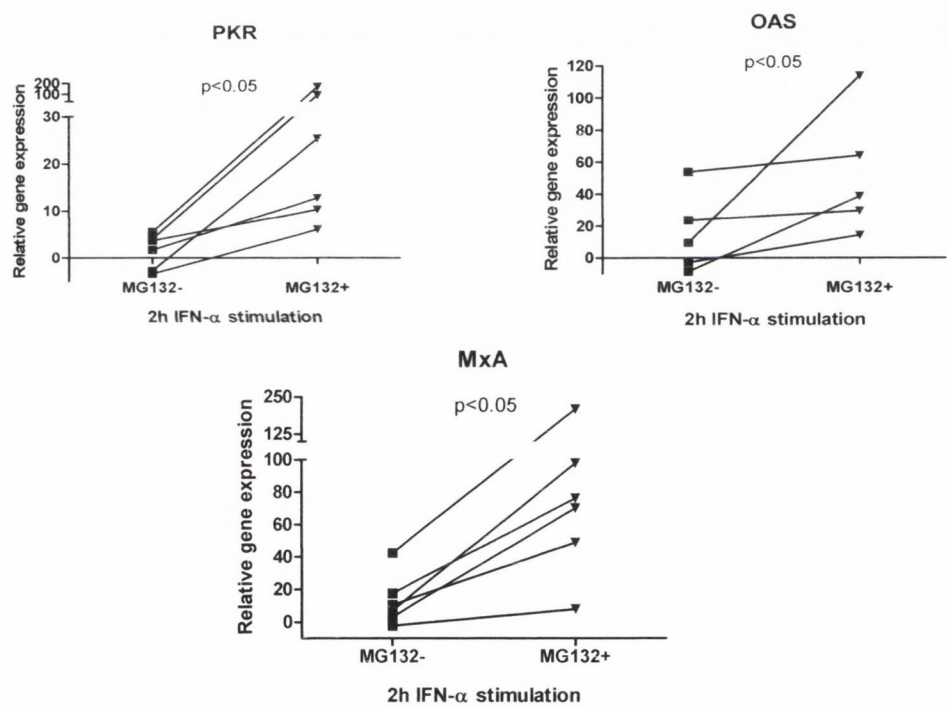


Figure 4.17. Proteasomal inhibition recovers ISG induction in PBMCs

PBMCs were isolated from HCV patients who failed to respond to clinical IFN- α therapy ($n=6$). 2×10^6 PBMCs were pre-treated +/- the proteasomal inhibitor MG132 (10 μ M) for 2h prior to stimulation with 1000IU IFN- α for 2h. Each sample is normalised to its endogenous RPS15 value and gene expression is relative to unstimulated control. Wilcoxon matched pairs analysis was used to compare individuals within the MG132- and the MG132+ groups.

4.2.15 STAT3 is required for optimal ISG induction in PBMCs

PBMCs from non-responder HCV patients have low STAT3 activation and low induction of anti-viral genes following stimulation with IFN- α (Fig. 4.9 and Fig. 4.12). Therefore we assessed whether STAT3 is required for ISG induction in PBMCs, as we had found it was necessary for ISG induction in Huh-7 cells (Fig. 3.9). We used siRNA to knock down STAT3 expression in PBMCs from healthy controls and quantified STAT3, PKR and MxA mRNA levels using qRT-PCR (Fig. 4.18). We found that STAT3 siRNA significantly knocks down STAT3 expression in PBMCs. Following treatment with STAT3 siRNA, PBMCs have reduced induction of the ISGs PKR and MxA compared to PBMCs treated with control siRNA.

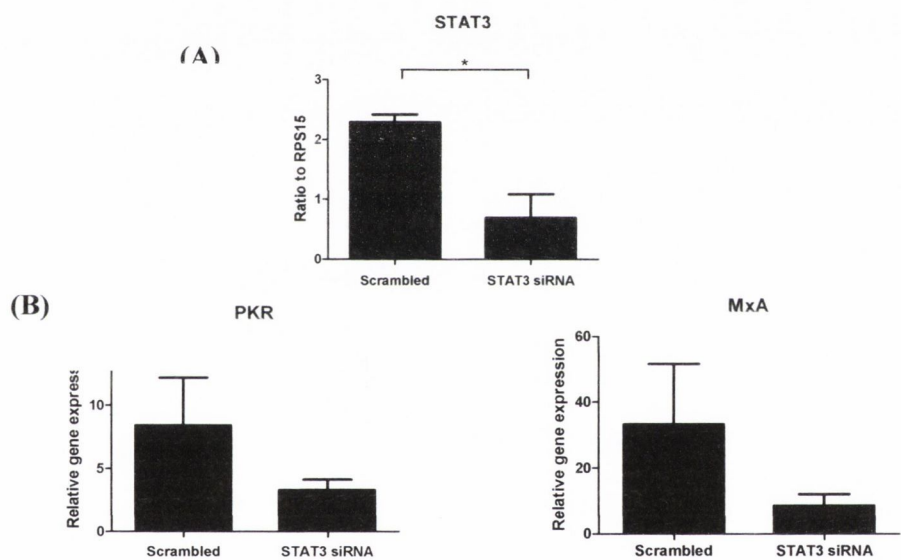


Figure 4.18. STAT3 is required for optimal ISG induction in PBMCs

(A) 2×10^6 PBMCs, isolated from healthy controls ($n=4$), were transfected with 60pmol of scrambled or STAT3 siRNA for 72h and qRT-PCR was used to quantify STAT3 mRNA expression. Gene expression levels relative to RPS15. (B) 2×10^6 PBMCs, isolated from healthy controls ($n=3$), were transfected with 60pmol of scrambled or native STAT3 siRNA for 72h and stimulated with 1000IU IFN- α for 2h. qRT-PCR was used to quantify PKR and MxA mRNA expression. All samples were normalised to RPS15 and gene expression was relative to unstimulated control. Wilcoxon matched pairs analysis statistical analysis was used.

4.2.16 STAT3 is required for the recovery of ISGs in non-responders

We found that ISG induction could be recovered in non-responder PBMCs following proteasomal inhibition (Fig. 4.17), and that STAT3 knock down resulted in compromised ISG induction in healthy PBMCs (Fig. 4.19), therefore we assessed if recovery of ISG induction in non-responder PBMCs was directly due to the recovery of STAT3 protein levels. As we previously found, treatment with MG132 could recover induction of both PKR and MxA (Fig. 4.19). However, when STAT3 expression was knocked down using siRNA, the non-responder PBMCs treated with MG132 failed to recover the induction of PKR, and to a lesser extent, MxA expression.

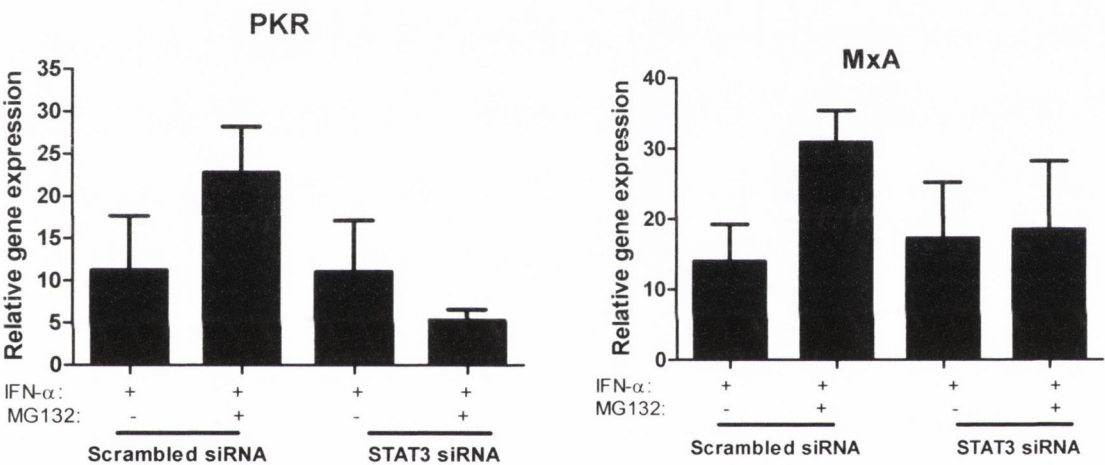


Figure 4.19. STAT3 is required for the recovery of ISGs via the proteasome in non-responder patients

2×10^6 PBMCs, isolated from non-responder patients ($n=3$), were transfected with 60pmol of scrambled or STAT3 siRNA for 72h, followed by 2h treatment with 10μM MG132 and then stimulated with 1000IU IFN-α for 2h. qRT-PCR was used to quantify expression of PKR and MxA mRNA. All samples were normalised to RPS15 and gene expression is relative to unstimulated control.

4.3 Discussion

Studies from our lab revealed that PBMCs from HCV infected patients lack expression of STAT1 and STAT3 proteins, key transcription factors involved in IFN- α signal transduction. Loss of STAT3 was seen in the PBMCs of 84% of HCV patients examined (16/19 patients all infected with genotype 1 of HCV, Dr. Nigel Stevenson, personal communication). IFN- α promotes the upregulation of many ISGs in PBMCs, both *in vivo* and *in vitro*, with many of these upregulated ISGs having anti-viral potential [198]. We propose that in PBMCs from HCV infected patients, absence of STAT1 and STAT3 results in reduced responsiveness in some patients to both endogenous and therapeutic IFN- α , explaining the low rate of spontaneous HCV clearance and lack of response to IFN- α therapy. Therefore the aim of this study was to examine the responsiveness of PBMCs from HCV patients to *in vitro* IFN- α stimulation. This would reveal the functional consequence of HCV-mediated degradation of STAT1 and STAT3 proteins within PBMCs and could reveal a important, novel role for STAT3 in IFN- α induced anti-viral gene expression, similar to its role in ISG induction in Huh-7 hepatocytes (Chapter 3).

As we wanted to study the *in vitro* responsiveness of PBMCs to IFN- α stimulation, we first determined the optimal duration of IFN- α stimulation required to upregulate ISG expression. As upregulation was similar at all time points examine, we concluded that 2h stimulation with IFN- α would be optimal for studying IFN- α responsiveness of PBMCs, as this short time would ensure that ISG induction was a direct effect of initial IFN- α stimulation and not due to a secondary, indirect effect activated by IFN- α stimulation.

IFN- α stimulated anti-viral gene expression patterns were similar in PBMCs from healthy controls and HCV patients; both groups contained individuals who displayed strong ISG induction after IFN- α stimulation whereas other individuals within both groups had poor upregulation of ISGs. Subtle variation in PBMC responsiveness may explain the diversity observed in clinical responsiveness to IFN- α therapy. Further analysis revealed that there were no significant differences in the endogenous basal levels of these genes in PBMCs from healthy and HCV infected

patients. This is different to what has been found in liver studies, where it was demonstrated that HCV infection in both human and chimpanzee results in higher hepatic ISG expression than in uninfected controls [214,215]. Interestingly, there was a lot of variance observed in endogenous ISG expression between individuals infected with HCV (Fig. 4.6). Constitutive upregulation of anti-viral ISGs would be predicted during viral infection. It is known that HCV targets RIG-I and TLR3 signalling to prevent activation of type I IFNs [135] and targets STAT1 to prevent IFN- α signalling [166]. Therefore, the ability of HCV to exert immunomodulatory effects such as these in some individuals may account for the variance observed in endogenous levels of ISGs within our patients.

Many factors such as age, sex and viral load can predict responsiveness to IFN- α therapy. A major viral factor that can determine overall response to therapy is viral genotype. Patients infected with genotype 1 of the virus have lower response rates to IFN- α therapy than any other genotype [2]. Therefore, we sought to determine whether those infected with genotype 1 had different responsiveness to IFN- α stimulation compared to those infected with other viral genotypes. Genotype non-1 infected patients showed diverse levels of OAS induction, but not of PKR and MxA. In contrast, genotype 1 infected patients showed diverse induction of all three anti-viral genes examined, with some patients having very high induction and others had very low induction. This was similar to the diversity observed in healthy controls. The versatile ISG induction in PBMCs from genotype 1 infected patients compared to genotype non-1 infected patients reflects the scenario in IFN- α therapy: that the vast majority of those not infected with genotype 1 respond similarly by clearing the virus, while those infected with genotype 1 have a diverse rate of response. In the genotype 1 infected patients, those with low ISG induction could be the patients whose STAT proteins are being targeted for proteasomal degradation. As we had found in chapter 3 that the binding sites that could allow HCV to act as an E3 ligase were not highly conserved in other viral genotypes, non-response to IFN- α may be via an alternative inhibitory mechanism in those infected with genotypes such as genotype 3. We also found that endogenous ISG expression was similar in genotype 1 and genotype non-1 infected patients. Interestingly, all HCV patients, regardless of viral genotype, had extremely diverse endogenous ISG expression in their PBMCs. This is in contrast to ISG

expression within the liver, where genotype 1 infected patients have been shown to have constitutively higher hepatic ISG expression than patients infected with other viral genotypes [205].

As we found that PBMCs from HCV patients showed a diverse range of *in vitro* ISG induction, we hypothesised that patients who failed to respond to IFN- α therapy would be the patients with poor induction of anti-viral genes *in vitro*. Therefore we analysed both endogenous ISG expression and ISG induction following IFN- α stimulation in the PBMCs from patients who had previously undergone IFN- α therapy and failed to respond; i.e. were still PCR +ve for the HCV. As controls, patients who had successfully responded to IFN- α treatment, and were PCR -ve for the virus, were used. We found significant differences in how these patients responded to *in vitro* IFN- α stimulation compared to the non-responders. All these patients were defined as non-responders as they were PCR +ve 6 months post treatment, further analysis is needed to measure ISG expression in non-responders based on their kinetic response to IFN- α therapy, as patients who were null responders (i.e. never responded to IFN- α at any time on treatment) may have very different *in vitro* ISG induction than patients who achieved partial response to treatment (i.e. achieved significant decline in HCV RNA titres while on treatment). Non-responder patients had very low ISG induction whereas the ISG induction in responders was significantly higher. Interestingly, endogenous ISG expression was similar in both groups. This finding contrasts with reports of hepatic ISG expression, as those least responsive to therapeutic administration of IFN- α have been shown to have higher hepatic ISG levels [6].

We showed that patients who had responded to therapy had significantly higher IFN- α stimulated anti-viral gene induction in their PBMCs than patients who had failed to respond. This difference was even more pronounced when those who had been infected with genotype 1 of the virus were examined. The stark difference in *in vitro* responsiveness to IFN- α stimulation could be indicative of the variation seen in the normal population and may correlate with clinical responsiveness to IFN- α therapy. The endogenous ISG expression in the non-responders is quite low compared to the endogenous levels in healthy and HCV -ve patients in Fig. 4.6, perhaps reflecting that HCV specifically suppresses endogenous ISG expression in the PBMCs of non-responders to avoid viral clearance. Another possible conclusion is that only induction

of the anti-viral ISGs is compromised. Indeed, it has been found that PBMCs from HCV infected patients have significantly elevated expression of SOCS3, the negative regulator of JAK-STAT signalling (Aideen Collins, unpublished data). Therefore, it would be interesting to measure SOCS3 expression in PBMCs from non-responder patients to investigate whether high SOCS3 expression was suppressing the endogenous expression of anti-viral ISGs.

As non-responders had low ISG induction following IFN- α stimulation *in vitro*, we wanted to investigate the JAK-STAT pathway in these patients. PBMCs from healthy controls and patients who had previously responded to therapy all showed strong phosphorylation of STAT1 and STAT3 following IFN- α stimulation. In contrast, non-responders to therapy showed little STAT1 and STAT3 activation, thus explaining why these patients exhibited such low ISG induction when stimulated. The decreased phosphorylation of STAT1 and STAT3 is most likely due to reduced protein expression of these transcription factors, as PBMCs from HCV infected patients have lower total STAT1 and STAT3 protein levels. The lack of STAT1 and STAT3 activation, and the subsequent failure to activate ISGs in response to IFN- α , may explain why these patients failed to respond to clinical IFN- α therapy.

Although we focused on analysing three classically activated ISGs upregulated by IFN- α , PKR, OAS and MxA, we also wanted to expand our study to analyse other IFN- α stimulated genes that may be of interest in HCV patients. High levels of the chemokine CXCL10 are found in HCV patients who ultimately fail to respond to IFN- α therapy [6], therefore we investigated CXCL10 mRNA levels in the PBMCs from patients who had previously undergone IFN- α therapy. We found that patients who did not respond to therapy had significantly higher expression of CXCL10 in their PBMCs, compared to patients who had responded. This difference in endogenous ISG expression between responders and non-responders was not what we had found for PKR OAS or MxA. Also in contrast to these ISGs, responders and non-responders had similar induction of CXCL10 mRNA following stimulation with IFN- α . This could be due to IFN- α activating alternative signalling pathways other than the JAK-STAT path, such as MAPK pathways [164]. Interestingly, it has been shown that the MAPK pathway upregulates CXCL10 expression in response to stimuli such as LPS and IFN- γ , therefore IFN- α could possibly be upregulating its expression in a similar manner [216].

Diversion of IFN- α signalling to alternative pathways may actually be a natural mechanism used by IFN- α to limit its own production of CXCL10 thereby controlling inflammation under strong IFN- α stimulation. This is also particularly interesting in light of recent findings that the high levels of CXCL10 seen in HCV patients may actually be the antagonist version of CXCL10 [217].

The E3 ligase Trim 21 is upregulated by IFN- α and limits the production of type I IFNs by targeting IRF3 and IRF7 for degradation via the proteasome [208]. This protein also has direct anti-viral activity against non-enveloped viruses by recognition of antibody coated viral particles in the cytoplasm of the cell and mediating their degradation [138,212]. As this had only been demonstrated for non-enveloped viruses, it is unlikely that Trim 21 possesses direct anti-viral activity against HCV, an enveloped virus. However, due to the important role Trim 21 plays in regulating type I IFN production, we wanted to investigate whether HCV could affect the expression of this E3 ligase. We found that in patients who did not respond to IFN- α therapy, endogenous Trim 21 mRNA expression was significantly higher than patients who had responded to therapy. There was no evidence of upregulation of Trim 19 mRNA. Interestingly, Trim 21 was similar to CXCL10, as the induction of both these genes following IFN- α stimulation was similar in both responders and non-responders despite significantly higher endogenous mRNA expression of both genes. Elevated Trim 21 expression possibly indicates a mechanism used by HCV to suppress the production of type I IFNs by manipulating a natural host mechanism for controlling signalling for its own advantage.

To ensure that any differences we observed were not due to differential levels of endogenous IFN- α or IFN- β , we measured basal levels of the mRNA for these proteins and also the mRNA levels following 2h IFN- α stimulation. We found that there was no significant difference in the endogenous levels of these genes, or their expression following IFN- α stimulation, between patients who had previously responded or failed to respond to therapy. This finding was unexpected as it has been previously demonstrated that PBMCs from HCV infected patients have significantly higher expression of IFN- α and IFN- β mRNA than PBMCs from healthy controls [218]. Further patients would need to be examined to provide conclusive data regarding type I IFN expression in PBMCs from responders and non-responders.

We had found that non-responders to therapy had low activation of STAT1 and STAT3 following IFN- α stimulation and ISG induction was compromised in these patients. Our lab had previously found that blocking the proteasome in PBMCs from HCV patients could recover STAT3 protein expression (Fig. 3.1); therefore we investigated whether proteasomal inhibition could recover ISG induction in non-responder PBMCs. Blocking the proteasome in non-responder PBMCs could significantly recover the induction of PKR, OAS and MxA. This observation, along with the results in Chapter 3 where small peptide inhibitors could block HCV degradation of STAT3 and recover ISG expression, indicate that specifically targeting the ability of HCV to degrade STAT proteins *in vivo* has potential to recover IFN- α responsiveness in patients who previously failed to respond to this therapy.

Although we showed that STAT3 was absent from patient PBMCs, and that MG132 could recover ISG induction in these cells, we could not directly link ISG recovery to STAT3 protein restoration. Although it had previously been shown in a cell line that STAT3 is essential for anti-viral gene induction [117], the role of STAT3 in ISG induction in PBMCs has never previously been assessed. Initially, we used STAT3 siRNA to examine the role of STAT3 induction in healthy PBMCs. We found that knock down of STAT3 expression resulted in decreased expression of PKR and MxA. Next, we set out to prove that the recovery of STAT3 in patient PBMCs following proteasomal inhibition was responsible for the increase in ISG expression. Treating cells with STAT3 siRNA and MG132 prior to IFN- α stimulation showed that ISG recovery was dependent on the recovery of STAT3 protein expression. STAT3 activation is known to induce anti-HCV activity *in vitro* [184], and our findings in patient PBMCs *ex vivo* further confirm the importance of STAT3 in anti-viral innate immunity and provide a rationale as to why HCV targets this transcription factor.

In conclusion, HCV targets STAT3 protein which compromises IFN- α signalling in PBMCs, which probably has major consequences both for effective viral clearance by the immune system and response to IFN- α therapy. PBMCs from HCV patients show diverse responses to *in vitro* IFN- α stimulation and this is particularly prominent between different HCV genotypes. Patients who failed to respond to IFN- α therapy had low ISG induction *in vitro* but could be made responsive to IFN- α in a STAT3 dependent manner by blocking the proteasome. The stark difference in ISG induction *in*

vitro between responders and non-responders indicated that studying ISG *in vitro* may be a powerful tool for discriminating patients who will respond clinically to IFN- α therapy from those who will fail to respond.

Chapter 5

Pre-treatment in vitro responsiveness to IFN- α as a predictor of clinical responsiveness

Chapter 5 Pre-treatment in vitro responsiveness to IFN- α as a predictor of clinical responsiveness

5.1 Introduction

HCV infection is treated with combination PEG-IFN- α and ribavirin. Success rates vary depending on the patient's viral genotype. Sustained virological response (SVR) is achieved in 40-50% of patients infected with HCV genotype 1 after 48 weeks of clinical IFN- α treatment, while patients infected with HCV genotypes 2 and 3 achieve SVR in 65-82% of cases after 24 weeks therapeutic IFN- α administration [2]. In patients infected with HCV, administration of IFN- α results in the rapid upregulation of ISGs in PBMCs but not in the liver [218,219]. Therefore, clinical IFN- α therapy acts on other sites in addition to the liver. To fully understand responsiveness to IFN- α therapy in HCV infected patients, a comprehensive examination of the effect of IFN- α on PBMCs is necessary. In particular, the response of PBMCs to IFN- α could be a useful indicator of physiological response to the cytokine and therefore a predictor of overall response to treatment. Additional investigations into the mechanisms of non-responsiveness to IFN- α therapy will allow for the development of more effective anti-viral drugs to treat HCV.

5.1.1 Viral factors

The most clearly defined baseline viral factor contributing to therapeutic non-response is viral genotype; patients infected with genotypes 2 and 3 have higher rates of SVR compared to genotype 1 infected patients [203]. Viral load can also have a significant impact on how a patient responds to treatment; patients with lower viral loads (<600,000 IU/ml) respond quicker to treatment and achieve higher rates of SVR [220]. An individual with high complexity of viral quasispecies is more likely to have a worse response to therapy than an individual with a small quasispecies repertoire [221]. The amino acid sequence in several HCV proteins have also been reported to influence

therapy outcome, with mutations in both core protein and NS5A protein being associated with treatment response [39,222].

5.1.2 Host factors

Many host factors have been identified that influence patient response to therapy. Women tend to have an increased chance of achieving SVR than men - due in part to the fact that women tend to drink less alcohol. Alcohol consumption accelerates fibrosis in HCV infected patients and the presence of bridging fibrosis is associated with a decreased likelihood of achieving SVR [223]. There is also an interesting link between women's endocrine system and treatment response. It has been recently found that post-menopause, women achieve SVR at a similar rate to men, and women who undergo an early menopause are less likely to respond to therapy [224]. Race has an important effect on treatment outcome, with black patients having lower SVR rates than non-black [225]. Other factors that predict non-responsiveness to therapy include high BMI, insulin resistance, excessive alcohol consumption and co-infection with other viruses [226]. Furthermore, as discussed previously in chapter 4, patients with high CXCL10 protein levels in their serum are less likely to respond to IFN- α therapy than those with lower CXCL10 pre-treatment serum levels.

Recently, several genome-wide association studies (GWAS) identified SNPs upstream of the IL28B gene that have a strong association with response to IFN- α therapy and also with spontaneous clearance of HCV [227,228]. The functional significance of these SNPs is still being elucidated. These IL28B related SNPs have been found to be independent of any other known predictors of response, including CXCL10 levels and viral mutations [229]. However although these SNPs are significantly linked to treatment response, one recent study showed that hepatic expression of ISGs is a stronger predictor of response than the IL28B SNPs [230]. This study calculated the relative predictive value of known predictors of non-response, including high hepatic ISG basal levels, the minor alleles of SNPs identified upstream of the IL28B gene, viral genotype and age. They found that the strongest predictor of non-response to therapy, regardless of viral genotype, was high hepatic ISG expression.

However, percutaneous liver biopsies are not without risk and there is a 1% incidence of serious complications including internal bleeding, infection and pain [231], therefore they do not represent a realistic option for predicting treatment response. A more practical option would be to identify a pre-treatment predictor of response using easily-accessible patient samples such as PBMCs.

5.1.3 HCV, PBMCs and IFN- α therapy

Microarray analysis on mRNA from patient PBMCs has been used to examine response to IFN- α during treatment. The *in vivo* and *ex vivo* responses of PBMCs to IFN- α have been demonstrated to be similar, indicating that *ex vivo* stimulation of PBMCs can be used to analyse physiological changes in IFN- α induced anti-viral gene expression [230]. Data from our lab have shown that the transcription factors STAT1 and STAT3 are degraded in PBMCs from HCV patients, and as described in the previous chapter, patients who previously responded to therapy have significantly greater ISG induction in their PBMCs following *in vitro* stimulation with IFN- α , compared with patients who failed to respond to therapy. Therefore, we hypothesised that those who fail to respond to therapy would fail to upregulate anti-viral genes in response to IFN- α pre-treatment. We predict this failure is due to lack of STAT proteins, as a result of viral degradation.

5.1.4 Disadvantages of IFN- α /ribavirin therapy

IFN- α /ribavirin therapy is a long, expensive course of treatment with severe side effects. IFN- α therapy is associated with influenza-like symptoms, including fatigue, headache and fever, and psychiatric side-effects including depression, which occur in up to 30% of those treated [232]. Other side effects include neutropenia and anaemia [233]. PEG-IFN- α is given subcutaneously once a week, with daily ribavirin being taken orally. Treatment duration depends on the viral genotype being treated; genotype 1 infected patients are treated for 48 weeks, whereas patients infected with genotype 2 or 3 are usually treated for 24 weeks. This treatment regime is mainly paid for by the

Irish Health Service Executive and costs €1950 per month. As an estimated 20,000-50,000 people are infected in Ireland [69], it is a significant economic burden to treat these patients. Therefore it is critical, both clinically and economically, to identify patients who are unlikely to respond to treatment, especially with the imminent introduction of new, more expensive treatment regimes which have additional side effects.

5.1.5 Future HCV treatment

Increased insight into the viral life cycle of HCV has allowed for the development of several new directly acting anti-viral agents. The drugs being phased into routine clinical use are based on small molecules that target the HCV NS3-4A protease, thus interfering with replication and polyprotein processing of the virus. It is also a particularly relevant target given that the NS3-4A protease is responsible for blocking many pathways of the innate immune response (Fig. 1.2). The two currently licensed NS3-4A protease inhibitors are telaprevir (Vertex) and boceprevir (Merck), both of which are used in combination with the standard IFN- α /ribavirin regime. In March of 2011, Merck published two trials on boceprevir: the SPRINT-2 and RESPOND-2 trials [234,235]. The SPRINT-2 trial evaluated boceprevir in 1,097 treatment naïve, genotype-1, mixed race HCV patients and found that the addition of boceprevir to IFN- α /ribavirin therapy significantly increased the SVR rates; in the nonblack cohort there was an increase in SVR from 40% to 60%; while black patients had an increase of 23% to 54% SVR [235]. The RESPOND-2 trial was a phase III trial which evaluated boceprevir in 403 genotype 1 infected patients who were previous partial responder or relapsers. Addition of boceprevir to IFN- α /ribavirin increased SVR rates from 24% to 68% for those of non-black race [234]. The number of black patients included in this trial was only 49 and SVR rates increased from 8% to 53% with triple therapy in this group, which is a significant improvement compared to IFN- α and ribavirin alone. However, it still results in non-response in almost half of black patients treated.

Following the publication of the boceprevir phase III trials, Merck published two trials on telaprevir in June 2011: the ADVANCE and the REALIZE trials. The ADVANCE trial evaluated 1,088 treatment naïve patients, infected with genotype 1, and found that the addition of telaprevir to the IFN- α /ribavirin treatment regime increased SVR rates from 44% to 75% [68]. In black patients (n=94 or 8% of total sample), SVR rates increased from 25% to 62% with addition of telaprevir to IFN- α /ribavirin therapy. The REALIZE trial evaluated 663 genotype 1 HCV patients who were previous null responders, partial responders or relapsers to IFN- α /ribavirin therapy who were being retreated with the addition of telaprevir [236]. Rates of SVR were significantly higher in telaprevir treated groups than the control group, with SVR rates increasing from 24% to 88%. Non-response to new protease inhibitors may be dependent on how an individual metabolises the drug. An important pathway in the metabolism of both telaprevir and boceprevir is the cytochrome CYP3A enzyme pathway [237]. However, metabolism rates for these drugs vary, with telaprevir having a plasma half life of 8-11h and boceprevir having a plasma half life of 3.5h. As high serum concentrations are needed to block the emergence of resistant mutants, variability, an important factor that could influence therapeutic failure to triple therapy may be CYP3A activity.

The success of these agents in enhancing response rates in genotype 1 infected patients will revolutionise treatment of HCV infection. However cost, adverse side effects and the emergence of drug resistant variants are all major challenges that need to be overcome. The additional adverse side effects of these protease inhibitors, along with the already unfavourable side effects that occur during IFN- α /ribavirin therapy, mean that some patients will not be able to tolerate triple therapy. In phase III trials of boceprevir and telaprevir, anaemia was seen in approximately 36% and 50% of patients respectively. This resulted in ribavirin dosage being reduced, as ribavirin can also cause anaemia, and during boceprevir treatment, erythropoietin had to be administered for up to 140 days in some cases. In Ireland, the use of these protease inhibitors is currently on a named patient basis. Of the three patients who have received Telaprevir to date in the Mater hospital Dublin, two required blood transfusions due to anaemia (Dr. Mary O'Neill, The Mater Hospital Dublin, personal communication). Rash, ranging from mild to severe, and cutaneous reactions are also common side

effects seen in more than 50% of patients taking telaprevir. A further drawback of these new drugs is they must be taken orally every 7 to 9 hours with high fat food for the entire regime. Failure to comply with therapy can lead to the emergence of viral mutants due to the existence of viral quasispecies within infected individuals. HCV mutants resistant to protease inhibitors have been demonstrated both *in vitro* and *in vivo* [238]. HCV NS3-4A protease requires very few viral amino acid substitutions to allow it to develop resistance to protease inhibitors [234,235]. Indeed, for the 30-40% of patients who fail to respond to triple therapy, this will hinder them from future therapeutic regimes due to the development of protease resistant mutants [239]. Another significant problem with the new protease inhibitors is the lack of response in up to half of black patients treated, meaning this therapy will be ineffective in a significant proportion of HCV infected individuals globally.

The success of these new viral specific compounds has paved the way for the development of other anti-HCV drugs, many of which have the potential to circumvent the need for the therapeutic use of IFN- α . New polymerase inhibitors, which target the NS5B RNA-dependent RNA polymerase, are in phase I and phase II clinical trials. These are divided into two classes: the nucleoside analogues, which interfere with the initiation of RNA transcription, and the non-nucleoside analogues, which bind directly to NS5B [240]. Other new therapies being developed include cyclophilin inhibitors, IFN- λ therapy and neutralising antibodies [241].

Dual therapy with IFN- α and ribavirin is an expensive treatment regime. In addition, IFN- α treatment is associated with neutropenia and ribavirin is associated with anaemia. Further costs can be €2,000 per month for G-CSF (for those suffering from neutropenia, given for approx 4 months) and €2,000 per month for erythropoietin (for those suffering from anaemia, given for approx 3 months). The new protease inhibitors are estimated to cost an additional €18,000 for one course of treatment. They further increase the risk of anaemia, which may require intervention with costly blood transfusion and EPO treatment. This ultimately results in triple therapy being too expensive for the vast majority of people infected with HCV globally. Moreover, as more than 20% of patients will fail to respond to the new therapeutics, it remains imperative to identify those non-responsive patients before they embark on this expensive, arduous regime. Identifying a negative predictor of response to IFN-

α /ribavirin will be very clinically relevant in the era of triple therapy. Therefore, a major aim of this study is to identify patients who would respond to IFN- α /ribavirin therapy, and which patients would benefit from the addition of protease inhibitors.

5.1.6 Patient response to treatment

Monitoring viral kinetics during treatment is a useful indicator as to whether a patient will ultimately respond to therapy (Table 5.1). Rapid virological response (RVR) is defined as being PCR –ve for HCV by week 4 of treatment. Early virological response (EVR) is defined as achieving a $>2 \log_{10}$ drop in viral load by week 12 of therapy. Patients who are PCR –ve at the end of treatment (EOT) have an EOT response. Overall response to therapy is determined 6 months after therapy is completed. Patients who are PCR –ve 6 months after treatment have a SVR. Patients who have an EOT response, but fail to have an SVR, are classed as relapsers.

Achieving RVR is a strong predictor of achieving overall SVR to therapy. Around 90% of patients who achieve RVR will achieve an SVR; however, patients who do not have an RVR but have an EVR still have a 65-72% of achieving SVR [226]. In contrast, failing the achieve EVR strongly predicts patients who will ultimately not have an SVR and patients infected with genotype 1 will have their treatment discontinued at week 12 if they have not achieved EVR [4]. Unfortunately, patients who fail to achieve SVR following IFN- α and ribavirin therapy are highly unlikely to respond to a second course of treatment. One study evaluated 20 patients who had failed to achieve SVR to initial IFN- α /ribavirin treatment, and were retreated with a second course of treatment and found that only 10% of the patients responded [242].

IFN-α therapy:	Week 4	Week 12	Week 24/48	6 months post tx
RVR	PCR -ve			
EVR		>2 log ₁₀ drop viral RNA		
EOT			PCR -ve	
SVR				PCR -ve
Relapser			PCR -ve	PCR +ve

Table 5.3. On-treatment response to IFN-α and ribavirin therapy

Patients who are PCR –ve at week 4 of treatment have a rapid virological response (RVR). Patients who achieve a >2 log₁₀ drop in viral RNA have an early virological response (EVR), patients who are PCR –ve at the end of treatment (EOT) have an EOT response. Patients who are PCR –ve 6 months after treatment completion have a sustained virological response (SVR). Patients who have an EOT response but experience viral breakthrough within the 6 months after treatment are classed as relapsers.

*Patients infected with genotype 1 and 4 are usually treated for 48 weeks; patients infected with other viral genotypes are typically treated for 24 weeks.

5.1.7 Aims

The objective of this prospective study was to identify signatures of *in vitro* responsiveness to IFN-α that might predict *in vivo* responsiveness to clinical IFN-α/ribavirin therapy. Not only would this predict patients who will not respond, identifying responders to standard IFN-α and ribavirin therapy would negate the need for intervention with protease inhibitors. To do this, we aimed to isolate PBMCs from HCV patients before they started therapy, stimulate the PBMCs with IFN-α *in vitro* and quantify expression of anti-viral ISGs. As infection with genotype 1 of HCV is the strongest predictor of treatment failure in HCV patients, we also wanted to compare *in vitro* PBMC responsiveness in genotype 1 patients and genotype non-1 infected patients, with respect to treatment outcome. We also aimed to examine ISG expression with on-treatment viral kinetics, as response to treatment at week 4 and week 12 of therapy are known on-treatment predictors of overall virological response.

5.2 Patient information

Patients recruited for this studying were all chronically infected with HCV and attending SVUH and SJH Hepatology units (Clinical characteristics of patients in this study are given in Table 5.2). They all commenced IFN- α treatment on the day of initial sample collection. Prior to the first administration of therapeutic IFN- α , 30mls of blood was taken. PBMCs were isolated from whole blood and 2×10^6 PBMCs/ml were stimulated *in vitro* with 100IU or 1000IU of IFN- α for 2, 4 and 18h. For this study, mRNA was extracted and cDNA was synthesised from cells stimulated for 2 and 4h. RNA extraction was unsuccessful from PBMCs of two patients (both genotype 1 infected). qRT-PCR was used to quantify mRNA expression of anti-viral genes. The results from these experiments were correlated with the response of the patients to treatment at various stages (Table 5.1).

	Total	Genotype 1	Genotype non-1	P value (g1 vs non-1)
Number	45	19	26 (G2:1; G3:23; G4:2)	1
Sex (M/F)	30/15	13/6	17/9	0.8371
Age (years -/+ SD)	42.8 -/+10.3	44.6 -/+ 13.2	41.7 -/+ 8	0.3774
ALT (IU/L -/+ SD)	111 -/+ 98	112.8 -/+ 122.6	109.4 -/+ 75.7	0.271
AST (IU/L -/+ SD)	70.2 -/+ 52	66.6 -/+ 54.8	73 -/+ 50.5	0.4411
WCC (x 10⁹/L -/+ SD)	6.2 -/+ 2.5	5.7 -/+ 2	6.7 -/+ 2.7	0.2657
Neut (x10³/μl)	3.3 -/+ 1.6	3.3 -/+ 1.4	3.3 -/+ 1.8	0.961
Lymph (x10³/μl)	2.2 -/+ 0.9	1.8 -/+ 0.6	2.5 -/+ 1	0.023
Mono (x10³/μl)	0.55 -/+ 0.25	0.46 -/+ 0.13	0.59 -/+ 0.3	0.105
Viral Load (x10⁶ IU -/+ SD)	7.3 -/+ 11.9	5.6 -/+ 10.8	8.6 -/+ 12.9	0.4414

Table 5.4. Summary of patient characteristics

A summary of clinical characteristics for total patients, also separated according to patients who were infected with genotype 1 and genotype non-1 of the virus. There was no significant difference in clinical characteristics between genotype 1 and genotype non-1 infected patients, with the exception of lymphocyte count as genotype 1 infected patients had significantly lower lymphocyte counts than genotype non-1 infected patients (p<0.05). ALT=Alanine Transaminase, a liver enzyme, individuals without liver disease have a normal range of 8-59 IU/L. AST=Aspartate Aminotransferase, a liver enzyme, individuals without liver disease have a normal range of 11-34 IU/L [243]. WCC=Whole Cell Count, Neut=Neutrophil count, Lymph=lymphocyte count, Mono=Monocyte count.

5.3 Results

5.3.1 Response rates to clinical IFN- α therapy

In total, 61% of the patients enrolled for this study achieved SVR (Table 5.3A & 5.3B). The lowest response rate was in patients who were infected with genotype 1 of HCV (56%), whereas those infected with other genotypes had a combined response rate of 65% (genotype 2, 100%, n=1; genotype 3, 65% n=20, genotype 4, 50%, n=2). Overall, 51% of patients achieved an RVR; 42% of genotype 1 infected patients and 58% for patients infected with other genotypes. 81% of patients had an EVR; 80% of genotype 1 infected and 82% of those infected with other genotypes. 79% of patients were PCR –ve at EOT; 72% of genotype 1 infected patients and 84% of those infected with other genotypes. Seven patients who were PCR –ve at the end of therapy had viral breakthrough within six months after treatment and these were classed as relapsers. Using Fisher’s exact test, we found no significant differences in RVR, EVR, EOT or SVR between genotype 1 infected patients and those infected with other viral genotypes.

Treatment response					
	Yes (N)	No (N)	Total (N)	% achieving response	
RVR	23	22	45	RVR	51
G1	8	11	19	G1	42
G2	1	0	1	G2	100
G3	13	10	23	G3	57
G4	1	1	2	G4	50
EVR	17	4	21	EVR	81
G1	8	2	10	G1	80
G3	9	1	10	G3	90
G4	0	1	1	G4	0
EOT	34	9	43	EOT	79
G1	13	5	18	G1	72
G2	1	0	1	G2	100
G3	19	3	22	G3	86
G4	1	1	2	G4	50
SVR	25	16	41	SVR	61
G1	10	8	18	G1	56
G2	1	0	1	G2	100
G3	13	7	20	G3	65
G4	1	1	2	G4	50

Table 5.3. Number of patients who achieved RVR, EVR, EOT and SVR
 Number, and corresponding %, of patients who achieved RVR, EVR, EOT and SVR. Each treatment time point was further analysed into patients infected with genotype 1 (G1), genotype 2 (G2), genotype 3 (G3) and genotype 4 (G4). 2 patients discontinued treatment after week 4 (1 G1 infected patients and 1 G3 infected patient). 2 more patients were not followed-up after treatment completion and therefore their SVR status is unknown; both were infected with G3.

5.3.2 Breakdown of patient response to treatment

Viral kinetics are monitored on treatment as they can predict the likelihood of achieving an SVR, therefore we analysed the predictive power of on-treatment viral kinetics within our patient cohort (Table 5.4). Regarding RVR, 82% of patients with an RVR achieved an SVR. RVR predicted SVR in 86% of genotype non-1 infected patients and 75% of patients infected with genotype 1. RVR was highly predictive of having an EVR (95%) and an EOT (91%). Not achieving RVR was associated with a 63% chance of not achieving SVR. Patients infected with genotype non-1 who did not achieve RVR were most likely not to have an SVR (67%); whereas 60% of genotype 1 infected patients who did not have an RVR did not have an SVR. In those without an RVR, most achieved an EVR (81%) and an EOT response (67%).

Having an EVR by week 12 of treatment was associated with a 67% chance of having an SVR, in total and for both genotype 1 and genotype non-1 infected patients. One of the two patients infected with genotype non-1 who failed to achieve EVR also failed to achieve an EOT response or an SVR.

To determine whether achieving RVR was a significantly better predictor of achieving SVR than EVR, Fisher's exact test was used and found that, although RVR could predict SVR in 82% of patients compared to EVR predicting it in 67%, this was not significantly different ($p=0.24$). Achieving an RVR was a strong positive predictor of EVR (PPV: 95%) but not achieving RVR could not predict not achieving EVR (NPV: 19%). Not achieving EVR has been shown to be strongly associated with not achieving an SVR, therefore genotype 1 infected individuals who did not achieve EVR ($n=3$) had their treatment discontinued at this point. 2 patients infected with genotype non-1 did not have an EVR, 1 patient achieved an SVR while the second patient did not. 78% of patients who were PCR -ve at EOT had an SVR, 77% of those infected with genotype 1 and 79% of patients infected with other viral genotypes. Patients who were PCR +ve at the end of treatment were PCR +ve 6 months after therapy.

		All	G non-1	G1
RVR	EVR	95% (21/22)	100% (14/14)	88% (7/8)
	EOT	91% (20/22)	100% (14/14)	75% (6/8)
	SVR	82% (18/22)	86% (12/14)	75% (6/8)
EVR	EOT	87% (33/38)	87% (20/23)	87% (13/15)
	SVR	67% (24/36)	67% (14/21)	67% (5/15)
EOT	SVR	78% (25/32)	79% (15/19)	77% (10/13)
		All	G non-1	G1
No RVR	No EVR	19% (4/21)	18% (2/11)	25% (2/8)
	No EOT	33% (7/21)	36% (4/11)	30% (3/10)
	No SVR	63% (12/19)	67% (6/9)	60% (6/10)
No EVR	No EOT	50% (1/2)	50% (1/2)	-
	No SVR	50% (1/2)	50% (1/2)	-
No EOT	No SVR	100% (9/9)	100% (4/4)	100% (5/5)

Table 5.4 Breakdown of response rates
 Predictive value for SVR based on achieving, or failing to achieve, RVR, EVR or EOT.

5.3.3 HCV patients (all genotypes) who achieve RVR have similar anti-viral gene expression pre-treatment as patients who do not achieve RVR

RVR is defined as undetectable HCV RNA by week 4 of treatment and is a strong predictor of SVR [244,245]. Therefore we analysed pre-treatment anti-viral gene expression using qRT-PCR, in PBMCs from pre-treatment patients of all viral genotypes, before and after stimulation with IFN- α *in vitro* for 2 and 4h and at a concentration of IFN- α of 100IU and 1000IU. Endogenous levels of PKR, OAS and MxA, before *in vitro* IFN- α stimulation, revealed that those who achieved RVR tended to have higher ISG expression pre-treatment, although this difference was not significant (Fig. 5.1 & Table 5.5). Patients who did not achieve SVR showed slightly higher ISG expression with 100IU IFN- α stimulation for 2h. This trend was similar with 1000IU IFN- α for 2h for OAS and MxA, but not PKR as PKR expression was slightly lower in those who did not achieve RVR than in those who did. Expression of ISGs was the same between patients with an RVR and those without an RVR with 100IU IFN- α stimulation for 4h. 4h stimulation with 1000IU IFN- α resulted in higher PKR and OAS expression in those with RVR, but was not significant (PKR, $p=0.06$; OAS, $p=0.07$). Expression of MxA after 4h stimulation with 1000IU IFN- α was similar in patients who achieved RVR and patients who did.

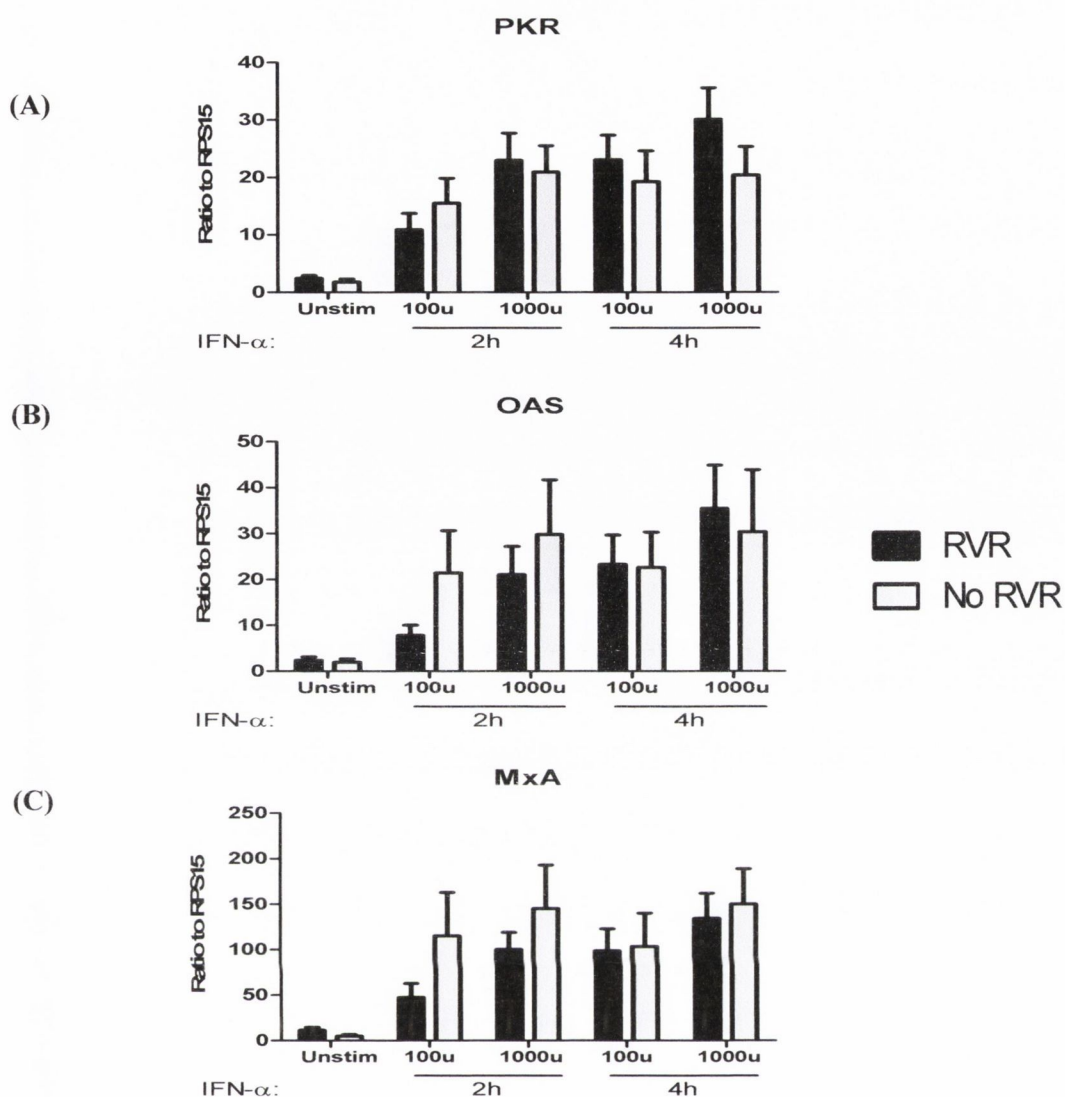


Figure 5.1. HCV infected patients (all genotypes) who achieve RVR have similar ISG expression pre-treatment as patients who fail to achieve RVR

PBMCs from HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved RVR and those who did not. RVR: control, n=21; 2h 100IU, n=17; 2h 1000IU, n=19; 4h 100IU, n=18; 4h 1000IU, n=21; no RVR: control, n=22; 2h 100IU, n=16; 2h 1000IU, n=17; 4h 100IU & 1000IU, n=21. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

All genotypes			RVR			No RVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		21	2.4	0.5	22	1.8	0.6	0.18	
	2h IFN-α	100IU	17	10.9	2.9	16	15.5	4.3	0.26	
		1000IU	19	23	4.7	17	21	4.6	0.86	
	4h IFN-α	100IU	18	24	4.4	21	19.3	5.4	0.21	
		1000IU	21	30.3	5.4	21	20.4	5	0.06	
OAS	Unstimulated		21	2.4	0.7	22	1.9	0.8	0.22	
	2h IFN-α	100IU	17	7.8	2.2	16	21.5	9.2	0.21	
		1000IU	19	21	6.1	17	29.8	11.9	0.28	
	4h IFN-α	100IU	18	23.3	6.3	21	22.7	7.7	0.83	
		1000IU	21	35.5	9.3	21	30.4	13.4	0.07	
MxA	Unstimulated		21	11.4	3.3	22	4.9	1.7	0.3	
	2h IFN-α	100IU	17	47.6	15.3	16	115.1	47.9	0.13	
		1000IU	19	100	18.9	17	145.3	47.7	0.83	
	4h IFN-α	100IU	18	98.7	24.2	21	103.4	36.8	0.2	
		1000IU	21	134.3	27.8	21	150.2	38.6	0.6	

Table 5.5. Summary of ISG expression and RVR: all genotypes
 Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all viral genotypes, analysed with respect to patients who achieved RVR and those who did not.

5.3.4 Genotype non-1 infected HCV patients who achieve RVR have similar pre-treatment ISG expression *in vitro* as those who fail to demonstrate RVR

In vitro ISG expression in PBMCs from pre-treatment genotype non-1 patients was analysed with respect to achieving RVR. Endogenous levels of PKR and MxA were slightly lower in patients who did not achieve RVR, whereas endogenous OAS levels were slightly elevated in the same patients (Fig. 5.2 and Table 5.6). Stimulation with IFN- α for 2h, at concentrations of either 100IU or 1000IU, showed no stronger upregulation of PKR, OAS and MxA in patients who subsequently did not achieve RVR than those who did. Stimulation for 4h with IFN- α , at concentration of 100IU or 1000IU, showed no significant difference in PKR, OAS or MxA between genotype non-1 patients who achieved RVR and those who did not. Overall, no significant trend emerged for RVR with respect to genotype non-1 infected patients and pre-treatment ISG expression.

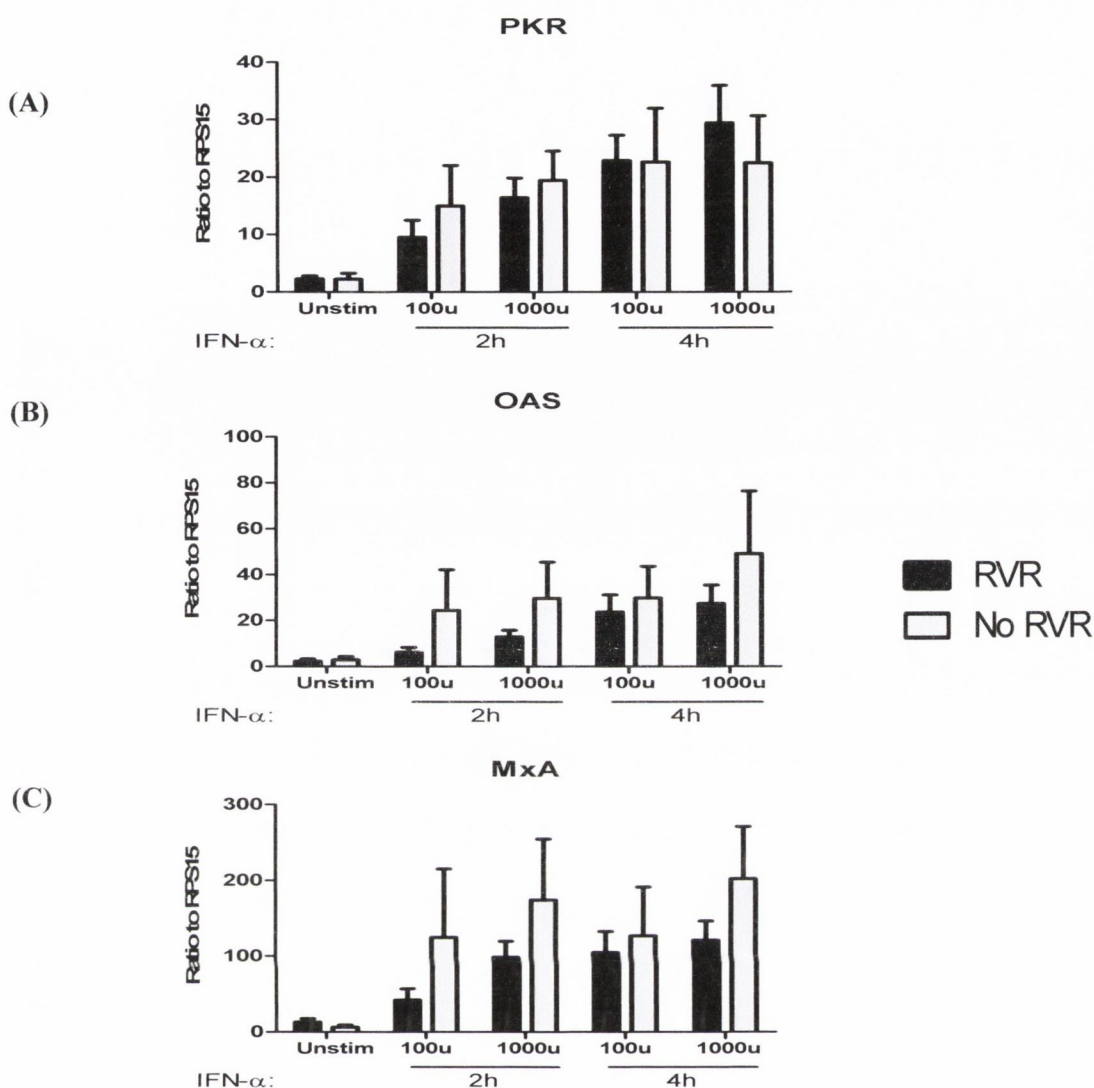


Figure 5.2. Genotype non-1 infected patients who achieve RVR have similar ISG expression pre-treatment as patients who fail to achieve RVR

PBMCs from genotype non-1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved RVR and those who did not. RVR: control, n=15; 2h 100IU & 1000IU, n=14; 4h 100IU & 1000IU, n=15; no RVR: control, n=11; 2h 100IU, n=8; 2h 1000IU, n=9; 4h 100IU, n=11; 4h 1000IU, n=10. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

Genotype non-1			RVR			No RVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		15	2.3	0.5	11	2.2	1	0.19	
	2h IFN-α	100IU	14	9.5	2.9	8	15	7	0.29	
		1000IU	14	16.4	3.4	9	19.5	5.1	0.64	
	4h IFN-α	100IU	15	22.9	4.4	11	22.6	9.4	0.3	
		1000IU	15	29.5	6.5	10	22.5	8.2	0.28	
OAS	Unstimulated		15	2.3	1	11	2.8	1.4	0.72	
	2h IFN-α	100IU	14	6.2	2.1	8	24.4	17.7	0.29	
		1000IU	14	12.8	3	9	29.8	15.7	0.73	
	4h IFN-α	100IU	15	23.8	7.5	11	29.9	13.8	0.96	
		1000IU	15	27.4	8	10	49.2	27.2	0.56	
MxA	Unstimulated		15	13.2	4.5	11	6.5	2.8	0.38	
	2h IFN-α	100IU	14	42.3	14.8	8	124.9	90.3	0.23	
		1000IU	14	98.5	21.2	9	174.3	80.4	0.97	
	4h IFN-α	100IU	15	104.5	28.1	11	126.8	64.7	0.28	
		1000IU	15	121.1	25.3	10	202.1	68	0.68	

Table 5.6. Summary of ISG expression and RVR: genotype non-1
 Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all viral genotypes except genotype 1, analysed with respect to patients who achieved RVR and those who did not.

5.3.5 Genotype 1 infected patients who achieve RVR have higher ISG expression in their PBMCs pre-treatment compared to patients who do not have RVR

Genotype 1 infected HCV patients have the highest failure rate to IFN- α therapy, therefore it is important to identify those who will respond and those who are likely to fail in this subset of patients. Achievement of RVR is a strong positive predictor of SVR, therefore we analysed *in vitro* responses at week 4 of treatment with pre-treatment ISG expression in PBMCs. Initially, we measured endogenous levels of PKR, OAS and MxA in pre-treatment PBMCs and analysed them with respect to RVR (Fig. 5.3 and Table 5.7). Patients who failed to achieve RVR had lower endogenous expression of both PKR and MxA than patients who did achieve RVR, and these patients also had significantly lower levels of OAS expression ($P<0.05$) in their PBMCs. 2h stimulation of PBMCs from patients who went on to achieve RVR with 100IU IFN- α showed higher expression of PKR than patients who did not have RVR, although expression of OAS and MxA was slightly less. MxA expression was also slightly less in these patients with 1000IU IFN- α , however this was not the case for OAS and PKR. No 2h stimulation with IFN- α showed any significant differences between those who achieved RVR and those who did not. 4h stimulation with 100IU IFN- α resulted in higher PKR and OAS expression in PBMCs from patients with RVR, whereas MxA expression was less. Stimulation for 4h with 1000IU IFN- α resulted in higher PKR, OAS and MxA expression in patients with RVR and this was significantly higher for OAS ($p<0.05$) compared to patients who did not go on to achieve RVR.

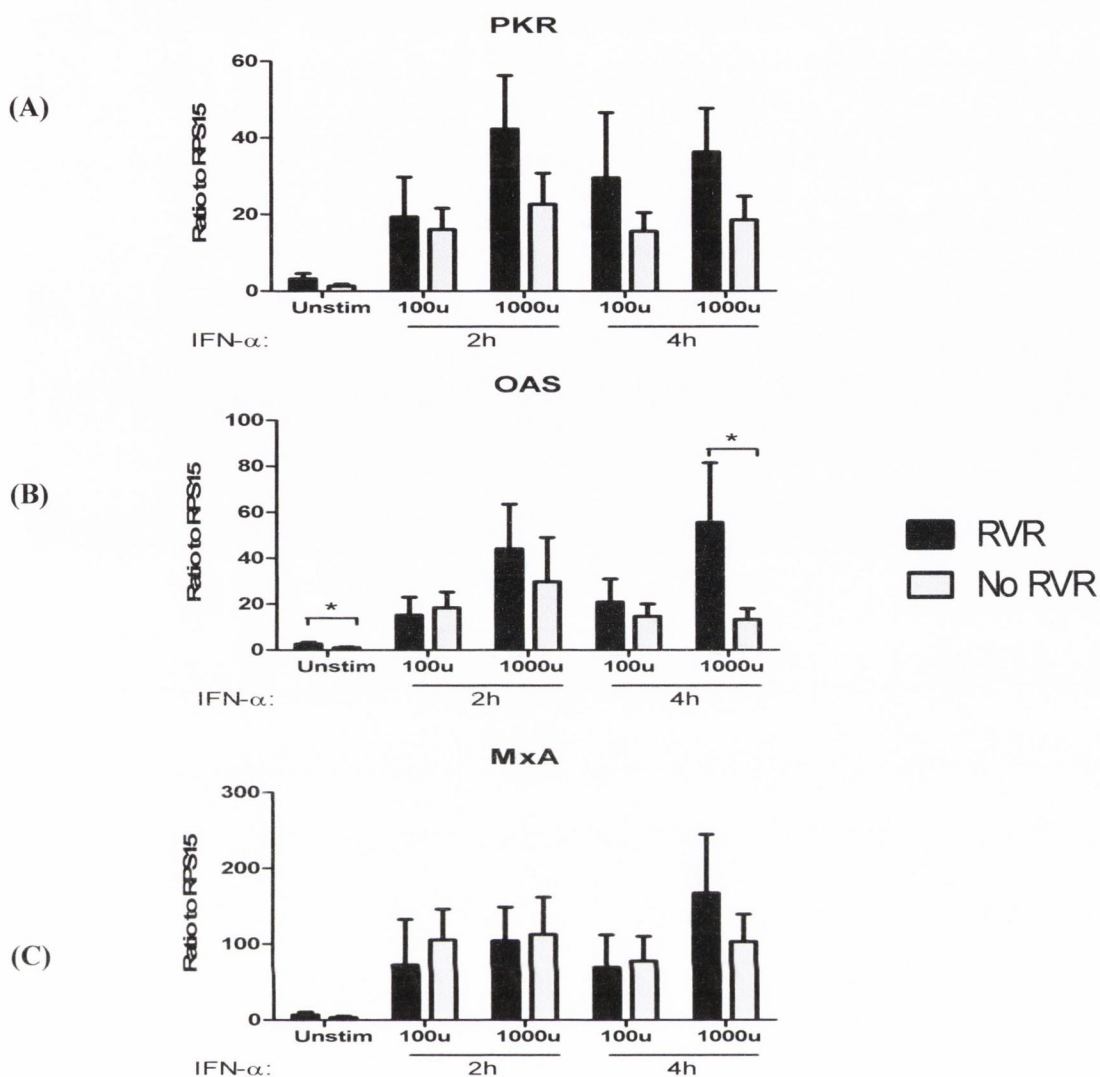


Figure 5.3. Genotype 1 patients who achieve RVR have higher pre-treatment ISG expression than patients who fail to achieve RVR

PBMCs from genotype 1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved RVR and those who did not. RVR: control, n=6; 2h 100IU, n=3; 2h 1000IU, n=5; 4h 100IU, n=3; & 1000IU, n=6; no RVR: control, n=11; 2h 100IU, n=8; 2h 1000IU, n=8; 4h 100IU, n=10; 4h 1000IU, n=11. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point. *p<0.05.

Genotype 1			RVR			No RVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		6	3.2	1.4	11	1.3	0.5	0.29	
	2h IFN- α	100IU	3	19.4	10.4	8	16.1	5.5	0.92	
		1000IU	5	42.3	13.9	8	22.6	8.2	0.22	
	4h IFN- α	100IU	3	29.6	17	10	15.6	4	0.57	
		1000IU	6	36.3	11.4	11	18.5	6.1	0.12	
OAS	Unstimulated		6	2.6	0.8	11	1	0.5	<0.05	Yes
	2h IFN- α	100IU	3	15.2	7.9	8	18.5	6.8	0.92	
		1000IU	5	44.1	19.4	8	29.8	19.3	0.13	
	4h IFN- α	100IU	3	20	9	10	14.7	5.4	0.69	
		1000IU	6	55.6	25.9	11	13.3	4.7	<0.05	Yes
MxA	Unstimulated		6	6.9	3.5	11	3.3	1.8	0.39	
	2h IFN- α	100IU	3	72.4	60.3	8	105.3	40.7	0.92	
		1000IU	5	104.2	44.6	8	112.6	49.4	0.83	
	4h IFN- α	100IU	3	69.5	42.6	10	77.6	32.6	0.57	
		1000IU	6	167.3	77.5	11	103	36.5	0.39	

Table 5.7. Summary of ISG expression and RVR: genotype 1
Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with genotype 1 of HCV, analysed with respect to patients who achieved RVR and those who did not.

5.3.6 HCV patients (all genotypes) who achieve EVR have higher ISG expression *in vitro* in their PBMCs pre-treatment

A patient is defined as having an EVR if they have a greater than 2 log₁₀ drop in viral load by week 12 of treatment. After RVR, it is the best on-treatment predictor of response. We aimed to determine whether there was a correlation between pre-treatment ISG expression in PBMCs and EVR. Patients who achieved an RVR were excluded from this analysis. Initially, we performed this analysis using data from patients infected with all genotypes combined. Patients who achieved EVR tended to have higher endogenous expression of ISGs in their PBMCs pre-treatment; although this was not significant (PKR, $p=0.62$; OAS, $p=0.5$; MxA, $p=0.3$) (Fig. 5.4 and Table 5.8). ISG expression following 2h stimulation with 100IU IFN- α was higher in patients with EVR than those without; this was significant for OAS expression ($p<0.05$) and although PKR and MxA expression tended to be higher in these patients, it was not significant. Stimulation with 1000IU IFN- α for 2h resulted in different levels of expression of the three anti-viral genes between those achieving EVR and those failing to achieve it, but with no significant trends emerging. 4h stimulation with IFN- α , with either concentrations, resulted in higher expression of PKR, OAS and MxA in patients with EVR, but this was not significant.

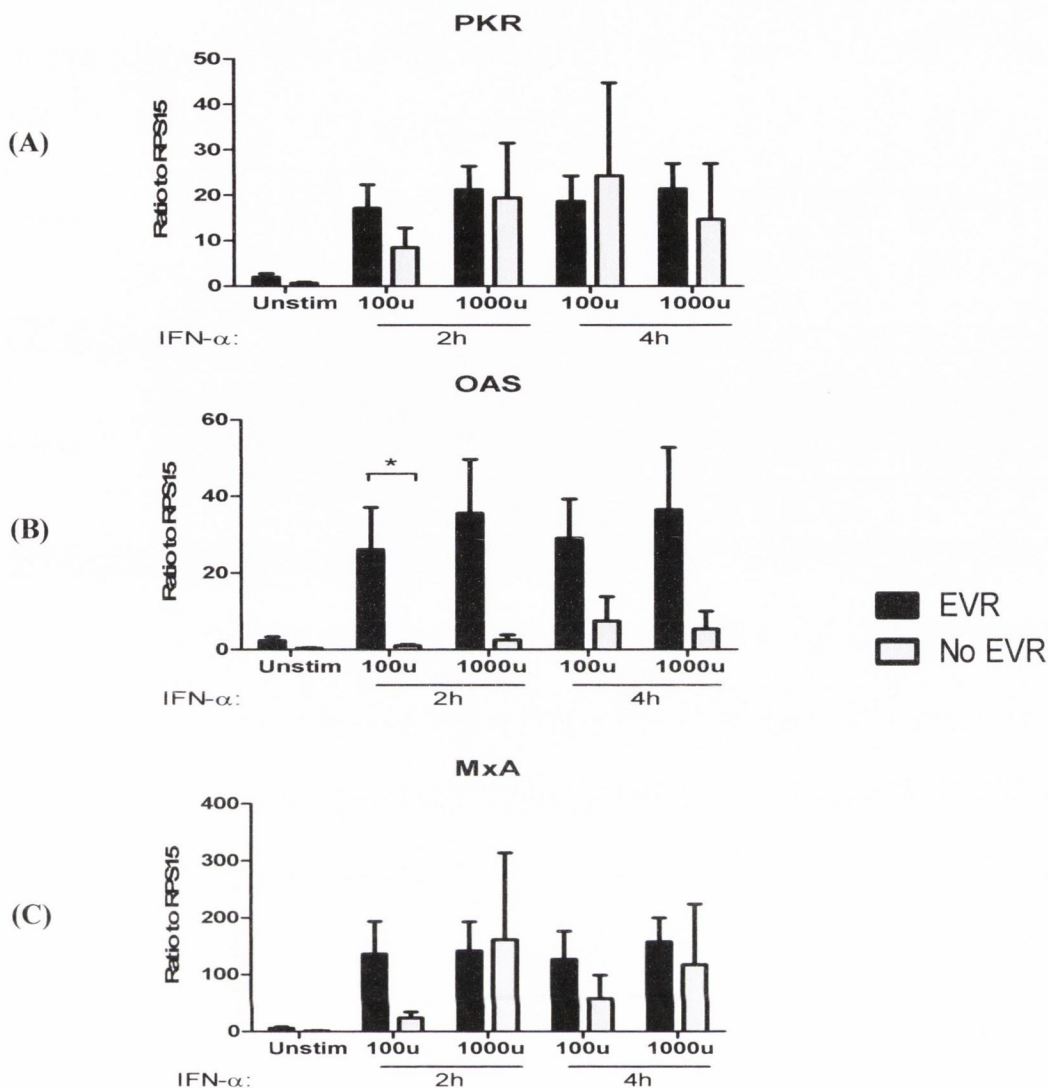


Figure 5.4. HCV infected patients (all genotypes) who achieve EVR have similar ISG expression pre-treatment as patients who fail to achieve EVR

PBMCs from HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved EVR and those who did not. EVR: control, n=17; 2h 100IU, n=13; 2h 1000IU, n=14; 4h 100IU, n=15; 4h 1000IU, n=17; no EVR: control, n=4; 2h 100IU & 1000IU, n=3; 4h 100IU & 1000IU, n=4. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

All genotypes			EVR			No EVR			p value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		17	2	0.7	4	0.7	0.2	0.62	
	2h IFN- α	100IU	13	17.2	5.2	3	8.5	4.3	0.42	
		1000IU	14	21.3	5.2	3	19.4	12.1	0.66	
	4h IFN- α	100IU	15	18.7	5.6	4	24.3	20.5	0.58	
		1000IU	17	21.4	5.6	4	14.7	12.3	0.45	
OAS	Unstimulated		17	2.4	1.0	4	0.4	0.1	0.50	
	2h IFN- α	100IU	13	26.2	11.0	3	1.0	0.4	0.01	Yes
		1000IU	14	35.6	14.1	3	2.5	1.3	0.15	
	4h IFN- α	100IU	15	29.0	10.3	4	7.5	6.3	0.09	
		1000IU	17	36.5	16.3	4	5.3	4.7	0.08	
MxA	Unstimulated		17	5.9	2.1	4	1.2	0.4	0.30	
	2h IFN- α	100IU	13	136.1	57.7	3	24.0	10.6	0.23	
		1000IU	14	141.8	51.2	3	161.3	152.5	0.57	
	4h IFN- α	100IU	15	126.9	49.6	4	57.7	41.1	0.73	
		1000IU	17	157.4	42.3	4	117.2	107.0	0.50	

Table 5.8. Summary of ISG expression and EVR: all genotypes
Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all viral genotypes, analysed with respect to patients who achieved EVR and those who did not.

5.3.7 Genotype non-1 infected patients who achieve EVR have similar ISG expression in their PBMCs pre-treatment as those who do not achieve EVR

82% of patients infected with HCV genotypes other than genotype 1 achieved EVR (Table 5.3). Therefore, analysis of this group is limited as the number of patients who did not achieve EVR is low (n=2), with both patients showing diverse *in vitro* response to IFN- α stimulation. The two patients who did not have EVR had lower endogenous expression of PKR, OAS and MxA in their PBMCs pre-treatment (Fig. 5.5 and Table 5.9). PKR expression following IFN- α stimulation for either 2h or 4h and with both concentrations, in the patients who did not have EVR was slightly higher than in patients who did have EVR. In these same patients, OAS expression was lower with all stimulations. MxA expression was lower for 2h and 4h stimulation with 100IU IFN- α in those without EVR but higher for 2h and 4h stimulation with 1000IU IFN- α .

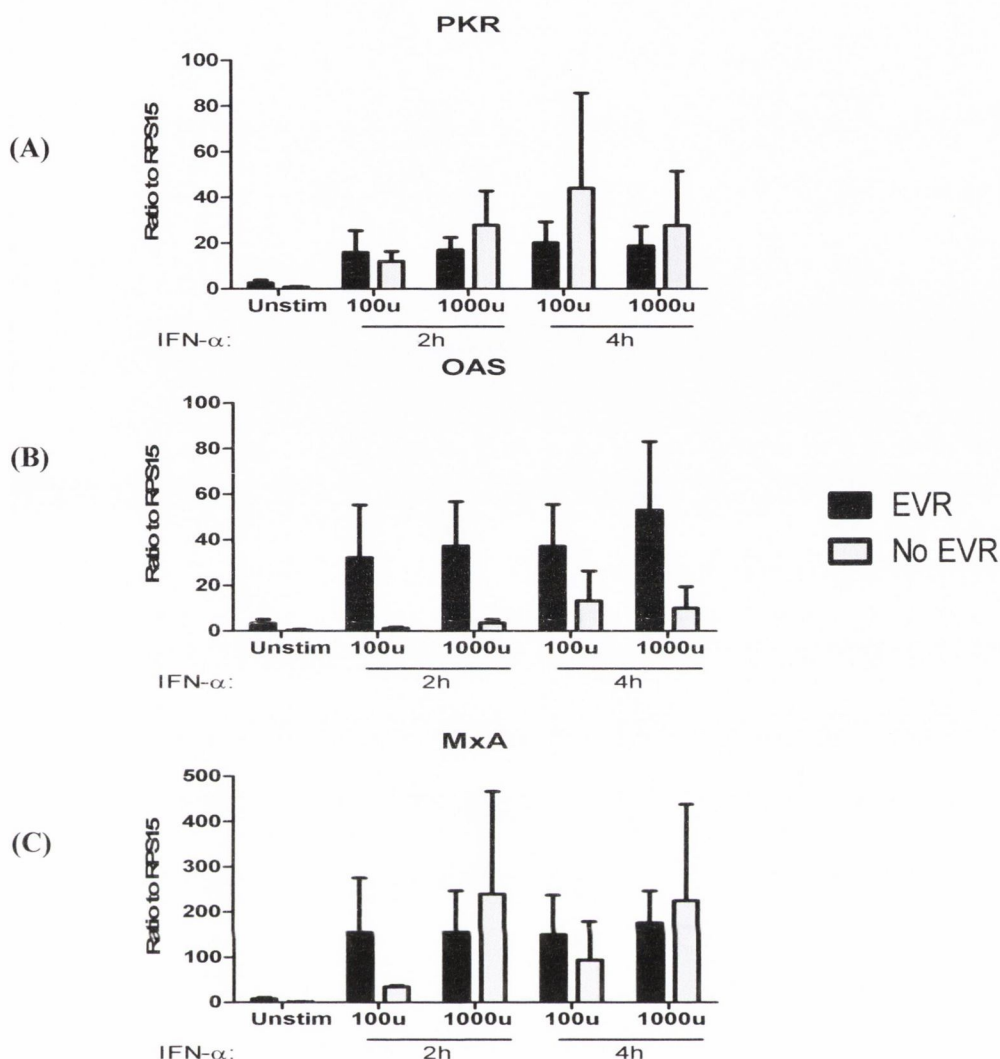


Figure 5.5. Genotype non-1 infected patients who achieve EVR have similar ISG expression pre-treatment as patients who fail to achieve EVR

PBMCs from genotype non-1 infected HCV patients were isolated prior to commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved EVR and those who did not. EVR: control, n=9; 2h 100IU, n=6; 2h 1000IU, n=7; 4h 100IU, n=8; 4h 1000IU, n=9; no EVR: control, 2h 100IU, 2h 1000IU, 4h 100IU & 4h 1000IU, n=2.

Genotype non-1			EVR			No EVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		9	2.5	1.2	2	0.8	0.2	N/A	
	2h IFN-α	100IU	6	16.0	9.5	2	12.0	4.3	N/A	
		1000IU	7	17.1	5.4	2	27.9	15.0	N/A	
	4h IFN-α	100IU	8	20.1	9.2	2	43.9	41.7	N/A	
		1000IU	9	18.8	8.5	2	27.7	23.7	N/A	
OAS	Unstimulated		9	3.4	1.7	2	0.4	0.3	N/A	
	2h IFN-α	100IU	6	32.2	23.2	2	1.2	0.6	N/A	
		1000IU	7	37.3	19.5	2	3.5	1.5	N/A	
	4h IFN-α	100IU	8	37.1	18.4	2	13.2	13.2	N/A	
		1000IU	9	53.0	30.1	2	9.9	9.5	N/A	
MxA	Unstimulated		9	7.7	3.3	2	1.0	0.7	N/A	
	2h IFN-α	100IU	6	155.0	120.4	2	34.5	2.8	N/A	
		1000IU	7	155.7	91.5	2	239.7	226.6	N/A	
	4h IFN-α	100IU	8	150.1	87.1	2	93.4	85.2	N/A	
		1000IU	9	175.4	71.4	2	224.6	213.4	N/A	

Table 5.9. Summary of ISG expression and EVR: genotype non-1
Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all viral genotypes except genotype 1, analysed with respect to patients who achieved EVR and those who did not.

5.3.8 Genotype 1 infected patients who achieve EVR have higher ISG expression pre-treatment in their PBMCs than patients who do not have EVR

Most patients infected with genotype 1 achieved EVR (80%, Table 5.3). Endogenous expression of PKR, OAS and MxA tended to be lower in patients without EVR; (Fig. 5.6 and Table 5.10). Due to the low patient number in the patients who failed to achieve EVR (n=2), statistical analysis could not be performed. For 2h stimulation with IFN- α , there was only gene expression data for 1 of the 2 genotype 1 infected patients with EVR, therefore Mann-Whitney analysis could not be performed on these groups. However, the patients without EVR tended to have lower expression of PKR, OAS and MxA in their PBMCs following IFN- α stimulation for 2h, particularly with 100IU IFN- α . 4h stimulation with 100IU and 1000IU of IFN- α resulted in higher ISG expression in patients with EVR than patients without an EVR.

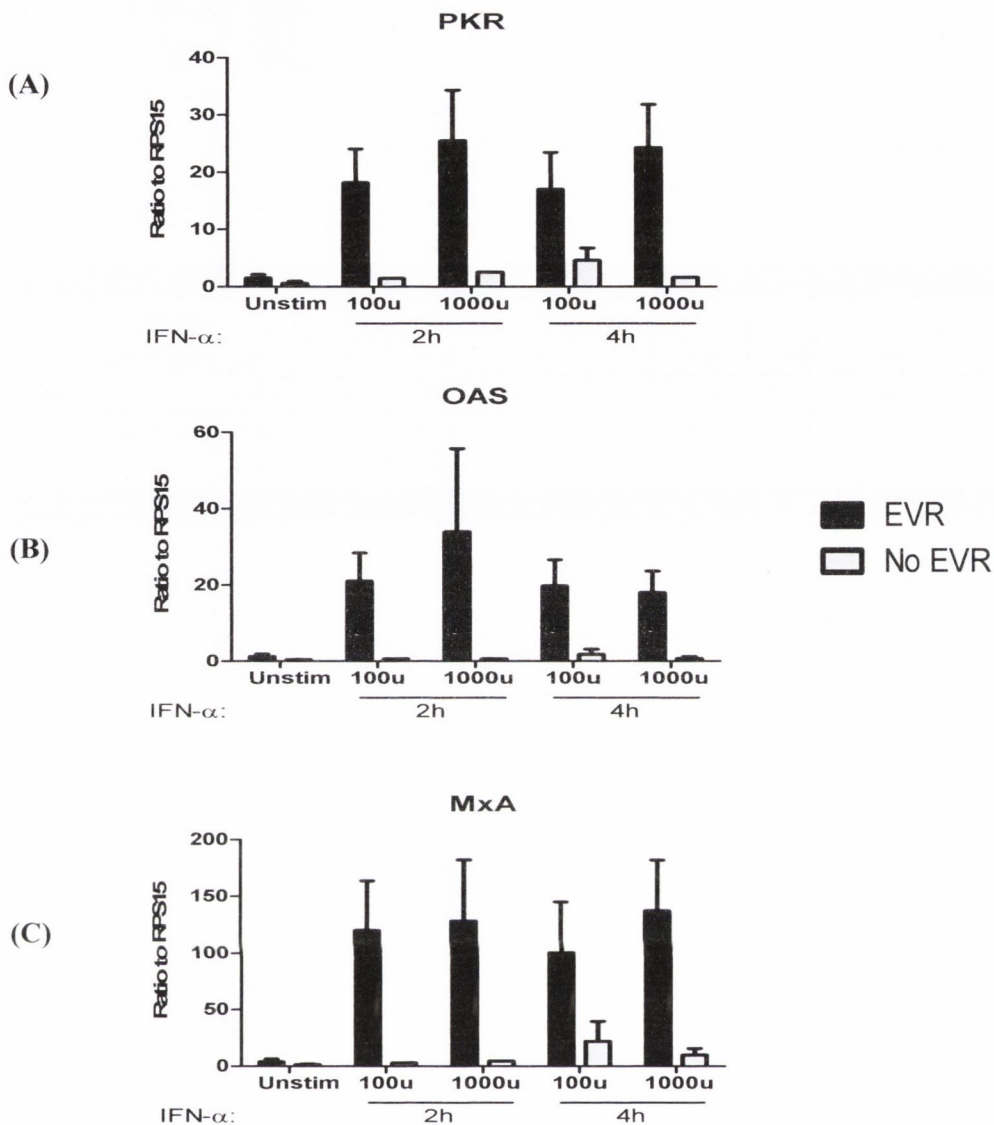


Figure 5.6. Genotype 1 patients who achieve EVR have higher pre-treatment ISG expression than patients who fail to achieve EVR

PBMCs from genotype 1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved EVR and those who did not. EVR: control, n=8; 2h 100IU, 2h 1000IU & 4h 100IU, n=7; 4h 1000IU, n=8; no EVR: control, n=2; 2h 100IU & 1000IU, n=1; 4h 100IU & 1000IU, n=2.

Genotype 1			EVR			No EVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		8	1.5	0.6	2	0.6	0.4	N/A	
	2h IFN- α	100IU	7	18.1	5.9	1	1.5	0.0	N/A	
		1000IU	7	25.5	8.8	1	2.5	0.0	N/A	
	4h IFN- α	100IU	7	17.0	6.4	2	4.6	2.2	N/A	
		1000IU	8	24.3	7.5	2	1.6	0.0	N/A	
OAS	Unstimulated		8	1.2	0.6	2	0.4	0.0	N/A	
	2h IFN- α	100IU	7	21.0	7.3	1	0.6	0.0	N/A	
		1000IU	7	33.9	21.8	1	0.6	0.0	N/A	
	4h IFN- α	100IU	7	19.7	6.8	2	1.8	1.4	N/A	
		1000IU	8	18.0	5.6	2	0.7	0.5	N/A	
MxA	Unstimulated		8	4.0	2.5	2	1.4	0.7	N/A	
	2h IFN- α	100IU	7	119.9	43.9	1	2.9	0.0	N/A	
		1000IU	7	128.0	54.1	1	4.5	0.0	N/A	
	4h IFN- α	100IU	7	100.4	44.5	2	22.0	17.6	N/A	
		1000IU	8	137.2	44.7	2	9.7	5.8	N/A	

Table 5.10. Summary of ISG expression and EVR: genotype 1
 Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with genotype 1, analysed with respect to patients who achieved EVR and those who did not.

5.3.9 ISG expression in pre-treatment PBMCs from patients of all genotypes does not predict who will be PCR –ve at the end of treatment

Treatment ended weeks for genotype 1 infected patients after 48 and after 24 weeks for those infected with other genotypes and patients were assessed to determine whether they were PCR –ve or PCR +ve for HCV. Patients who were PCR –ve at the end of treatment tended to have higher expression of PKR, OAS and MxA pre-treatment, although the p values showed that this was far from significant and therefore definite differences cannot be assumed (Fig. 5.7 and Table 5.11). Stimulation of pre-treatment PBMCs for 2h with 100IU or 1000IU showed higher expression of PKR, OAS and MxA in patients who subsequently were PCR +ve at the end of treatment. Expression of PKR in these patients was lower compared to PCR –ve patients at all 4h IFN- α stimulations. Expression of OAS was higher in those who were still PCR +ve at all 4h stimulations, as was MxA expression following 100IU IFN- α stimulation for 4h. MxA expression tended to be lower pre-treatment following 1000IU IFN- α stimulation in PCR +ve patients.

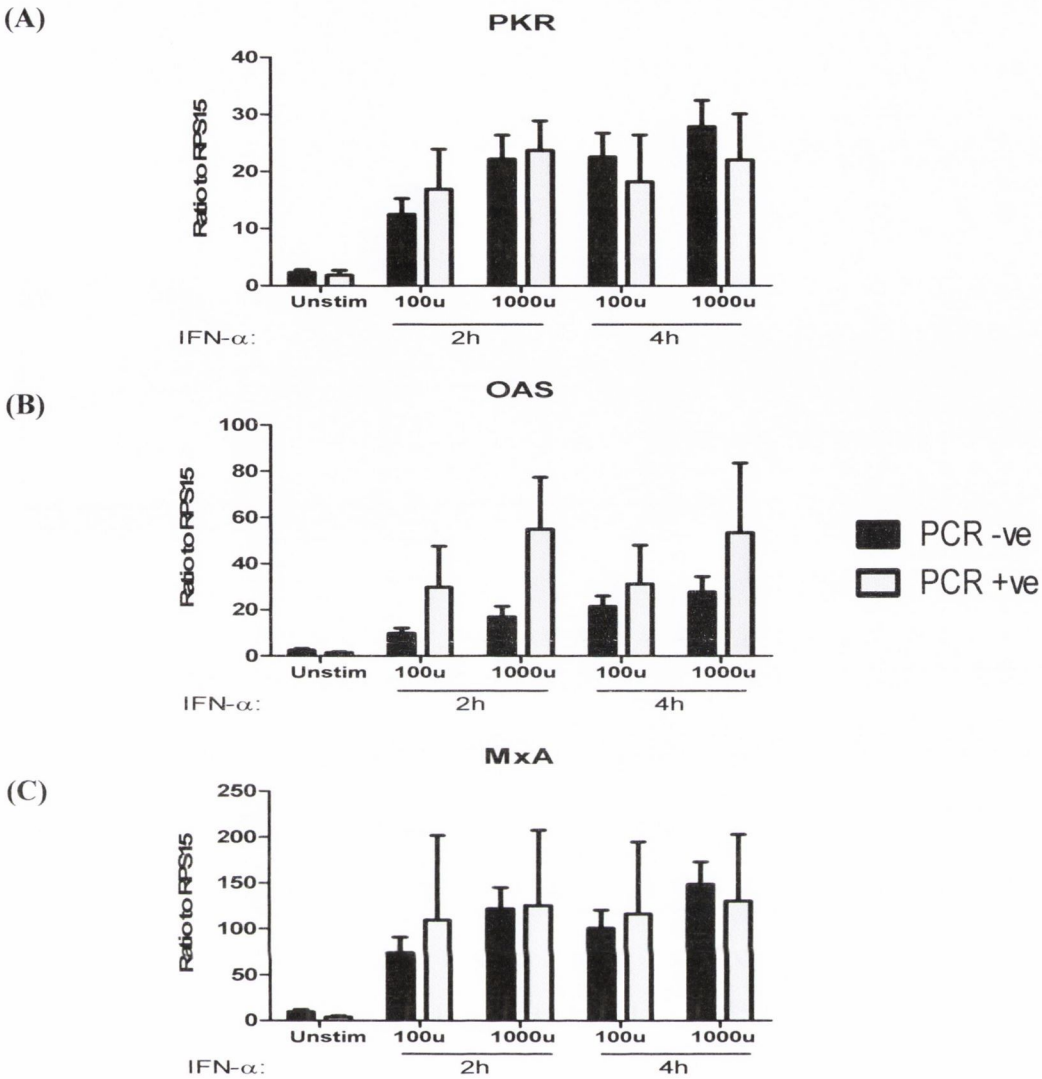


Figure 5.7. HCV infected patients (all genotypes) who are PCR –ve and PCR +ve at the end of treatment have similar ISG expression pre-treatment

PBMCs from HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression data was segregated according to those who were PCR –ve at treatment end and those who were not. PCR +ve: control, n=32; 2h 100IU, n=24; 2h 1000IU, n=27; 4h 100IU, n=28; 4h 1000IU, n=31; PCR -ve: control, n=9; 2h 100IU & 1000IU, n=8; 4h 100IU & 1000IU, n=9. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

All genotypes			PCR -ve			PCR +ve			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		32	2.3	0.5	9	1.8	0.9	0.81	
	2h IFN-α	100IU	24	12.5	2.7	8	17	7	0.59	
		1000IU	27	22.2	4.2	8	23.7	5.2	0.42	
	4h IFN-α	100IU	28	22.6	4.2	9	18.2	8.2	0.58	
		1000IU	31	27.9	4.5	9	22	8.1	0.48	
OAS	Unstimulated		32	2.485	0.7	9	1.4	0.4	0.89	
	2h IFN-α	100IU	24	9.878	2.2	8	29.8	17.8	0.54	
		1000IU	27	16.97	4.6	8	55	22.4	0.25	
	4h IFN-α	100IU	28	21.57	4.5	9	31.3	16.8	0.72	
		1000IU	31	27.76	6.7	9	53.4	30.1	0.54	
MxA	Unstimulated		32	9.69	2.4	9	3.761	1.6	0.36	
	2h IFN-α	100IU	24	73.87	17.1	8	109.4	92.3	0.2	
		1000IU	27	121.7	23.3	8	125.4	82	0.18	
	4h IFN-α	100IU	28	100.5	19.7	9	116.2	78.7	0.25	
		1000IU	31	148.6	24.4	9	130.3	72.6	0.15	

Table 5.11. Summary of ISG expression and EOT: all genotypes
Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all viral genotypes, analysed with respect to patients who achieved EOT response and those who did not.

5.3.10 Genotype non-1 infected patients who are PCR +ve at the end of treatment have higher ISG expression in their PBMCs pre-treatment

ISG expression pre-treatment was analysed in PBMCs from genotype non-1 infected patients and assessed with regards to PCR status at the end of treatment. Endogenous levels of PKR expression were lower pre-treatment in patients who were PCR –ve at the end of treatment (Fig. 5.8 and Table 5.12). In contrast, patients who were PCR –ve had slightly higher endogenous levels of OAS and MxA pre-treatment. Stimulation of pre-treatment PBMCs, from genotype non-1 infected patients, with 100IU or 1000IU IFN- α , for 2 or 4h, resulted in higher expression of PKR, OAS and MxA in the patients were PCR +ve at the conclusion of IFN- α therapy.

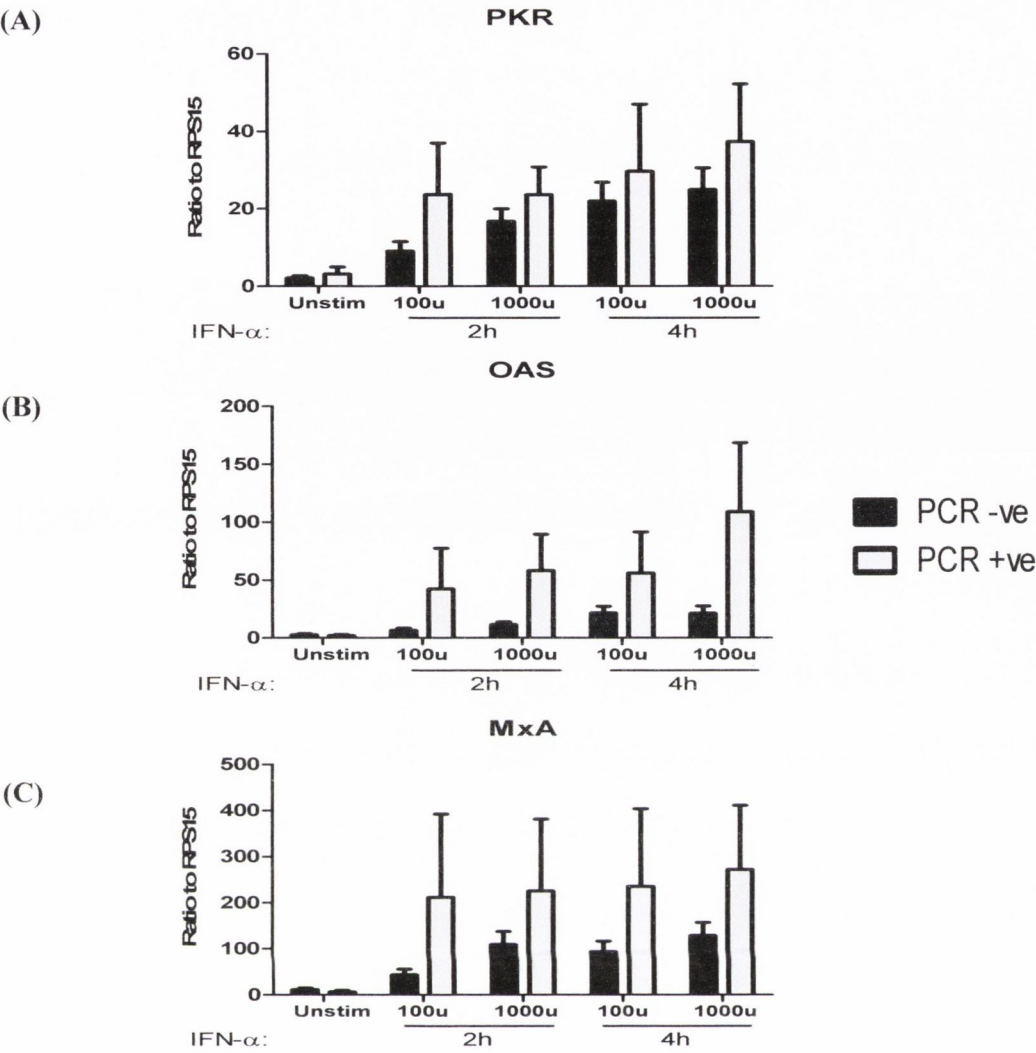


Figure 5.8. Genotype non-1 infected patients who are PCR –ve or PCR +ve at the end of treatment have similar ISG expression pre-treatment

PBMCs from genotype non-1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who were PCR –ve at the end of treatment and those who were not. PCR -ve: control, n=21; 2h 100IU, n=17; 2h 1000IU, n=18; 4h 100IU, n=21; 4h 1000IU, n=20; PCR +ve: control, 2h 100IU, 2h 1000IU, 4h 100IU & 4h 1000IU, n=4. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

Genotype non-1			PCR -ve			PCR +ve			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		21	2.2	0.5	4	3.2	1.8	0.39	
	2h IFN-α	100IU	17	9.1	2.5	4	23.7	13.2	0.17	
		1000IU	18	16.7	3.3	4	23.6	7.2	0.33	
	4h IFN-α	100IU	21	21	4.8	4	29.6	17.4	0.85	
		1000IU	20	24.9	5.6	4	37.3	14.9	0.42	
OAS	Unstimulated		21	2.7	1	4	2.1	0.7	0.48	
	2h IFN-α	100IU	17	6.6	1.8	4	42.5	35.1	0.28	
		1000IU	18	11.5	2.5	4	58.2	31.4	0.47	
	4h IFN-α	100IU	21	21.8	5.6	4	56	35.6	0.63	
		1000IU	20	21.4	6.2	4	108.8	59.6	0.37	
MxA	Unstimulated		21	11.5	3.5	4	6.6	3	0.97	
	2h IFN-α	100IU	17	43.6	12.3	4	211.7	181	0.35	
		1000IU	18	109.2	28	4	225.6	155.8	0.7	
	4h IFN-α	100IU	21	93.5	22.8	4	235.7	168.2	0.68	
		1000IU	20	128.6	28.1	4	272	139.1	0.33	

Table 5.12. Summary of ISG expression and EOT: genotype non-1
 Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all HCV genotypes except genotype 1, analysed with respect to patients who achieved an EOT response and those who did not.

5.3.11 Genotype 1 infected patients who are PCR –ve at the end of treatment have higher ISG expression in their PBMCs pre-treatment

PBMCs from genotype 1 infected patients, who were PCR –ve or PCR +ve at the end of treatment, were analysed for ISG expression. Endogenous expression of anti-viral genes in the PBMCs of these patients followed the same trend: patients who were PCR –ve at the end of treatment had higher endogenous levels of PKR, OAS and MxA pre-treatment whereas patients who were subsequently PCR +ve at treatment end had lower expression of these genes in their PBMCs pre-treatment (Fig. 5.9 and Table 5.13). Stimulation with 100IU IFN- α for 2h resulted in higher expression of PKR, OAS and MxA in the PBMCs of the patients who were PCR –ve at the end of treatment compared to those who were PCR +ve but this difference was only significant for MxA ($P<0.01$). Stimulation with 1000IU IFN- α for 2h resulted in higher PKR and MxA expression in those who went on to be PCR –ve after treatment, again MxA expression was the only gene that was significantly higher ($p<0.05$). In contrast, OAS expression tended to be lower in these patients. ISG expression pre-treatment following 4h stimulation with 100IU IFN- α was higher in those who were PCR –ve at the end of treatment but this was not significant. Stimulation with 1000IU IFN- α for 4h resulted in significantly higher expression of PKR, OAS and MxA in those who went on to be PCR -ve at the end of treatment compared to those who remained PCR +ve ($p<0.05$, $p<0.05$ and $p<0.01$ respectively).

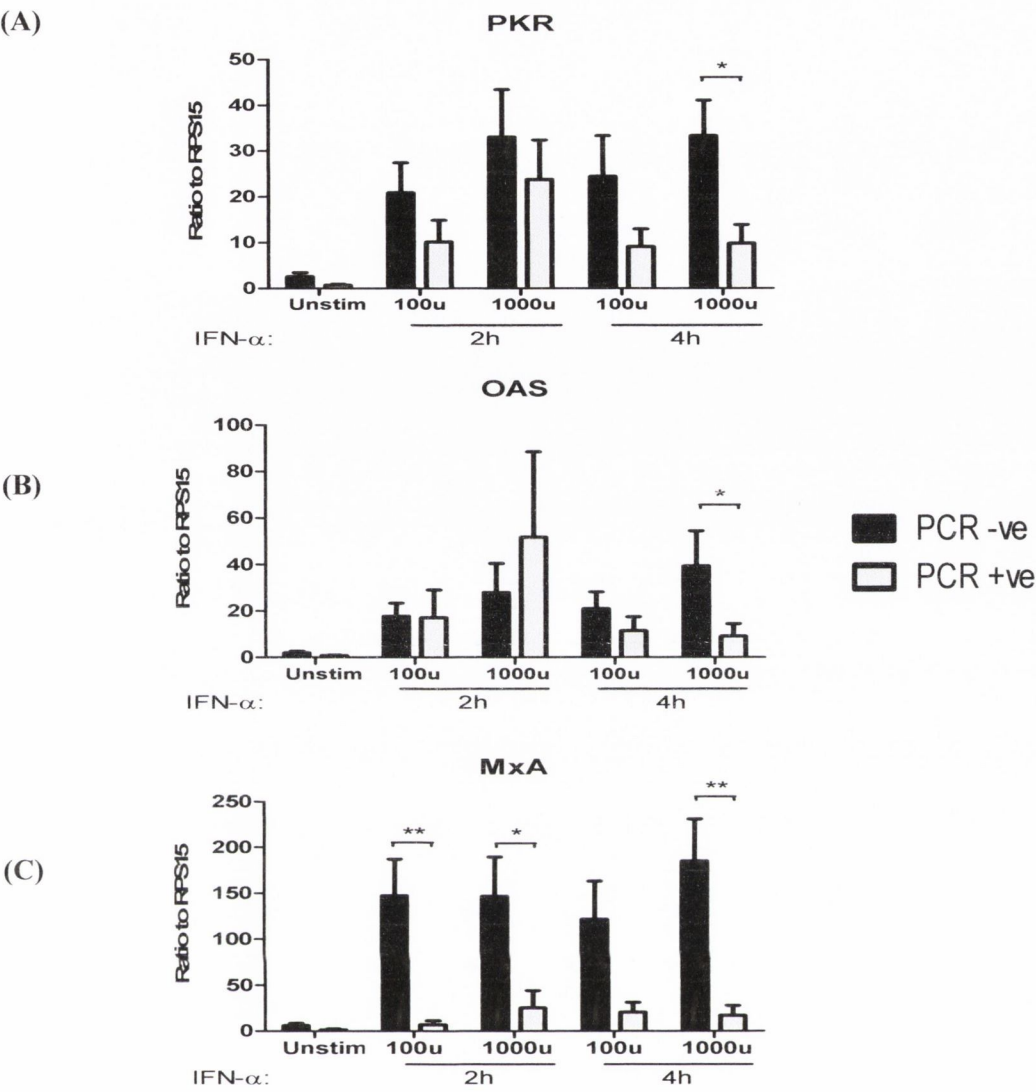


Figure 5.9. Genotype 1 patients who are PCR –ve at the end of treatment have higher pre-treatment ISG expression than patients who remain HCV +ve

PBMCs from genotype 1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who were PCR –ve at end of treatment and those who were not. PCR -ve: control, n=11; 2h 100IU, n=7; 2h 1000IU, n=9; 4h 100IU, n=7; 4h 1000IU, n=11; PCR +ve: control, n=5; 2h 100IU & 1000IU, n=4; 4h 100IU & 1000IU, n=5. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.*p<0.05, **p<0.01.

Genotype 1			PCR -ve			PCR +ve			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		11	2.6	0.8	5	0.7	0.2	0.17	
	2h IFN-α	100IU	7	20.9	6.6	4	10.1	4.7	0.41	
		1000IU	9	33.1	10.3	4	23.8	8.6	0.83	
	4h IFN-α	100IU	7	24.5	8.8	5	9.1	3.8	0.43	
		1000IU	11	33.3	7.8	5	9.8	4.1	<0.05	Yes
OAS	Unstimulated		11	2	0.6	5	0.8	0.2	0.57	
	2h IFN-α	100IU	7	17.8	5.6	4	17.1	11	0.79	
		1000IU	9	27	12.5	4	51.8	36.7	0.6	
	4h IFN-α	100IU	7	21	7.2	5	11.5	5	0.34	
		1000IU	11	39.4	15	5	9.1	5.4	<0.05	Yes
MxA	Unstimulated		11	6.2	2.5	5	1.5	0.8	0.14	
	2h IFN-α	100IU	7	147.3	39.9	4	7	4.3	<0.01	Yes
		1000IU	9	146.8	42.7	4	25.1	19	<0.05	Yes
	4h IFN-α	100IU	7	121.7	41.5	5	20.5	10.7	0.07	
		1000IU	11	185	45.7	5	16	10.8	<0.01	Yes

Table 5.13. Summary of ISG expression and EOT: genotype 1
 Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with HCV genotype 1, analysed with respect to patients who achieved an EOT response and those who did not.

5.3.12. Patients (all genotypes) who achieve an SVR tend to have higher ISG expression in their PBMCs pre-treatment

Overall response to IFN- α therapy is determined 6 months after treatment; if a patient is PCR –ve at this point, they have achieved an SVR. As this is the overall response to treatment, this category is most relevant regarding association with pre-treatment ISG expression. Initially, ISG expression and SVR was analysed in patients of all viral genotypes. Patients who achieved SVR tended to have higher pre-treatment expression of PKR, OAS and MxA in their PBMCs pre-treatment than patients who did not have SVR (Fig. 5.10 and Table 5.14). Higher levels of PKR and MxA expression after IFN- α stimulation was seen in patients who achieved SVR compared to those who did not, however although this was consistent for all three anti-viral genes, it was not significant. OAS expression was lower in those who had SVR than those who did not for all 2h IFN- α stimulations and also for 4h stimulation with 1000IU IFN- α . 4h stimulation with 100IU IFN- α resulted in slightly lower OAS expression in patients who subsequently did not attain SVR.

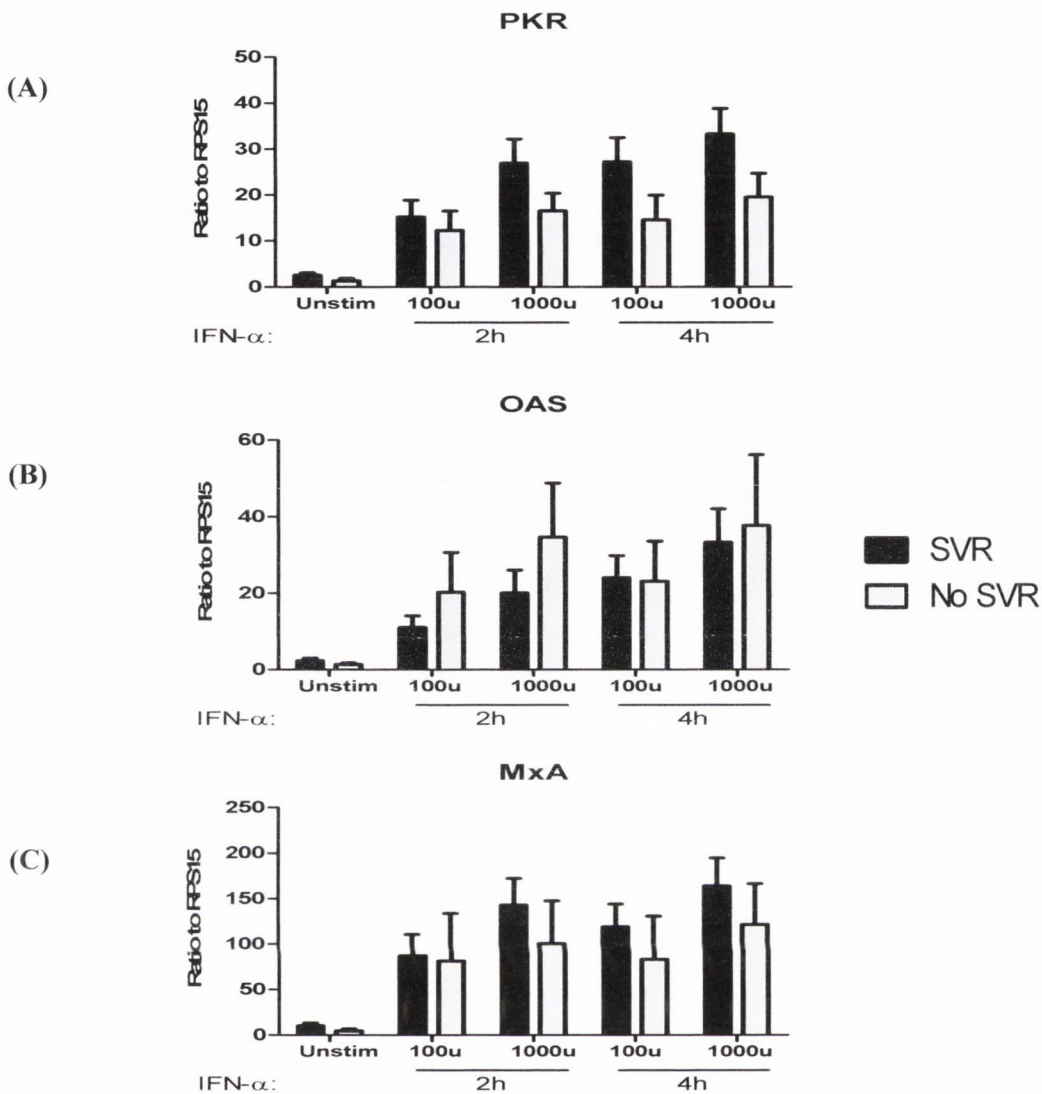


Figure 5.10. HCV infected patients (all genotypes) who achieve SVR have similar ISG expression pre-treatment as patients who fail to achieve SVR

PBMCs from HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved SVR and those who did not. SVR: control, n=23; 2h 100IU, n=17; 2h 1000IU & 4h 100IU, n=20; 4h 1000IU, n=23; no SVR: control, n=16; 2h 100IU & 1000IU, n=14; 4h 100IU & 1000IU, n=15; 4h 1000IU. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

All genotypes			SVR			No SVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		23	2.549	0.5106	16	1.37	0.4966	0.14	
	2h IFN- α	100IU	17	15.22	3.652	14	12.31	4.211	0.74	
		1000IU	20	26.94	5.241	14	16.51	3.847	0.29	
	4h IFN- α	100IU	20	27.23	5.228	15	14.55	5.392	0.07	
		1000IU	23	33.28	5.537	15	19.54	5.173	0.1	
OAS	Unstimulated		23	2.343	0.6989	16	1.372	0.3662	0.68	
	2h IFN- α	100IU	17	11.04	3.033	14	20.26	10.4	0.62	
		1000IU	20	20.07	6.003	14	34.68	14.08	0.93	
	4h IFN- α	100IU	20	24.08	5.753	15	23.15	10.5	0.37	
		1000IU	23	33.31	8.696	15	37.67	18.48	0.21	
MxA	Unstimulated		23	10.28	3.06	16	4.735	1.808	0.24	
	2h IFN- α	100IU	17	87.45	23.13	14	81.74	52.21	0.26	
		1000IU	20	143	29.39	14	100.5	47.03	0.09	
	4h IFN- α	100IU	20	119	25.2	15	83.37	47.44	0.08	
		1000IU	23	164	30.58	15	121.5	45.14	0.21	

Table 5.14. Summary of ISG expression and SVR: all genotypes
Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all viral genotypes, analysed with respect to patients who achieved SVR and those who did not.

5.3.13. There is no correlation with pre-treatment ISGs in PBMCs and SVR in genotype non-1 HCV patients

Patients infected with genotypes other than genotype 1 have a high rate of response to IFN- α therapy. Patients infected with genotype non-1 who achieved SVR had similar endogenous expression levels of PKR, OAS and MxA in their PBMCs before treatment than those who didn't achieve SVR (Fig. 5.11 and Table 5.15). PKR expression was slightly lower in those with SVR than those without for 2h stimulation with 100IU IFN- α and both concentrations at 4h, whereas for 2h stimulation with 1000IU IFN- α , expression of PKR was slightly higher in patients with SVR. OAS and MxA followed the same trend for all IFN- α stimulations: patients who achieved SVR had slightly less OAS and MxA expression post stimulation than patients who did not have SVR. Again, none of these slight differences showed any levels of significance and were only general trends observed. A summary of pre-treatment ISG expression in PBMCs from genotype non-1 infected patients is given in table 5.16.

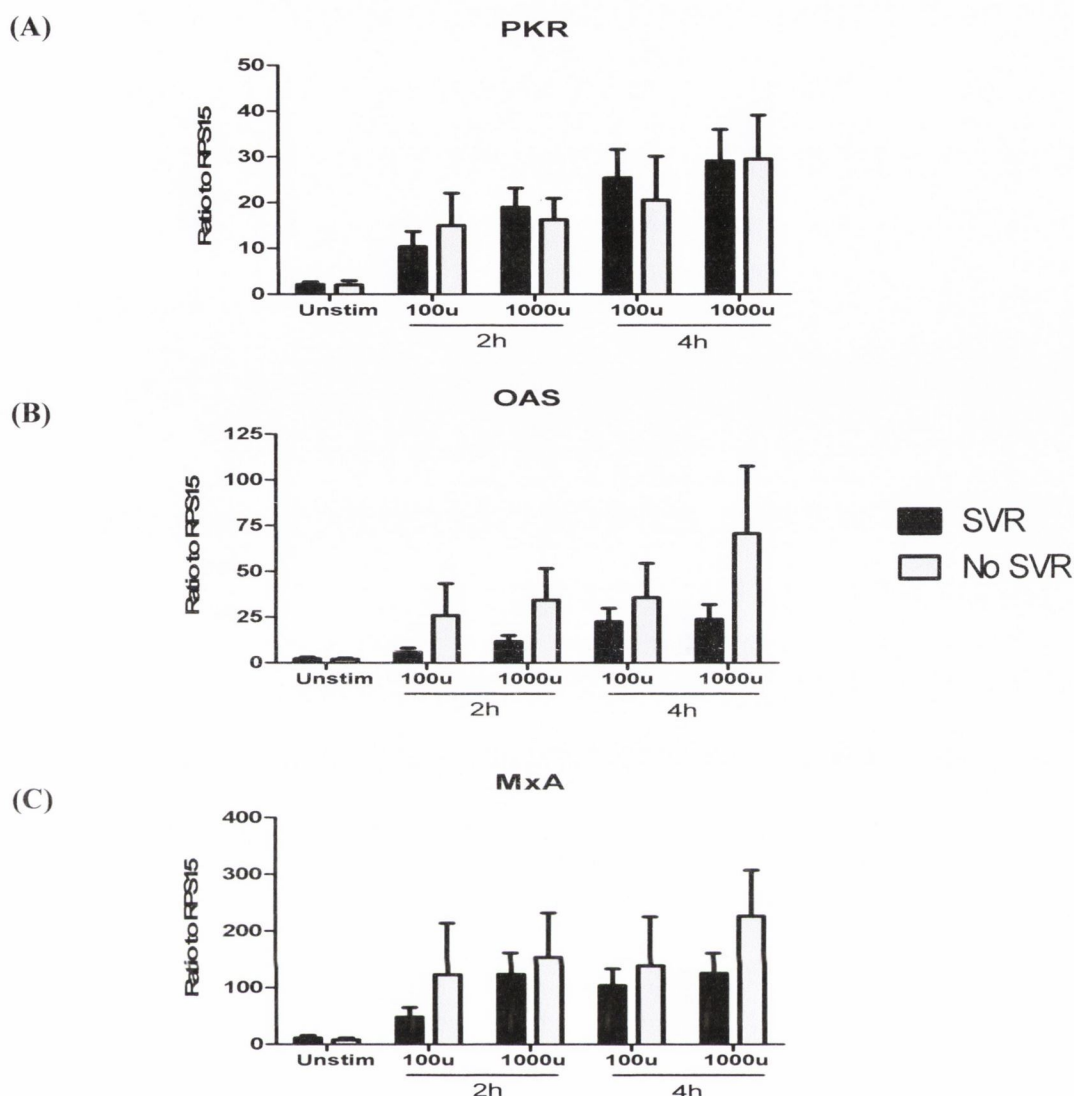


Figure 5.11. Genotype non-1 infected patients who achieve SVR have similar ISG expression pre-treatment as patients who fail to achieve SVR

PBMCs from genotype non-1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved SVR and those who did not. SVR: control, n=15; 2h 100IU, n=12; 2h 1000IU, n=13; 4h 100IU & 1000IU, n=15; no SVR: control, 2h 100IU & 1000IU, 4h 100IU, n=8; 4h 1000IU, n=7. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

Genotype non-1			SVR			No SVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		15	2.1	0.5	8	2	1	0.87	
	2h IFN-α	100IU	12	10.36	3.3	8	15	7.1	0.42	
		1000IU	13	19	4.2	8	16.3	4.6	0.8	
	4h IFN-α	100IU	15	25.4	6.2	8	20.6	9.6	0.5	
		1000IU	15	29.1	6.9	7	29.5	9.6	0.94	
OAS	Unstimulated		15	2.2	1	8	2	0.6	0.63	
	2h IFN-α	100IU	12	5.9	2.2	8	25	17.5	0.1	
		1000IU	13	11.2	3.3	8	34.5	17.2	0.59	
	4h IFN-α	100IU	15	22.6	7.3	8	35.7	18.7	0.87	
		1000IU	15	23.9	8	7	70.6	36.8	0.53	
MxA	Unstimulated		15	11.4	4.4	8	8.1	3.2	0.92	
	2h IFN-α	100IU	12	48.6	16.7	8	123.6	90.5	0.73	
		1000IU	13	124.3	37.1	8	153.7	78.4	0.97	
	4h IFN-α	100IU	15	104.2	29.8	8	139	86.3	0.97	
		1000IU	15	126	34	7	226.4	81.2	0.18	

Table 5.15. Summary of ISG expression and SVR: genotype non-1
 Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with all viral genotypes except genotype 1, analysed with respect to patients who achieved SVR and those who did not.

PKR										OAS										MxA									
RVR					No RVR					RVR					No RVR					RVR					No RVR				
n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value					
Unstim	15	2.3 (0.5)	11	2.2 (1)	0.19	15	2.3 (1)	11	2.8 (1.4)	0.72	15	13.2 (4.5)	11	6.5 (2.8)	0.38														
2h 100u	14	9.5 (2.9)	8	15 (7)	0.29	14	6.2 (2.1)	8	24.4 (17.7)	0.29	14	42.3 (14.8)	8	124.9 (90.3)	0.23														
2h 1000u	14	16.4 (3.4)	9	19.5 (5.1)	0.64	14	12.8 (3)	9	29.8 (15.7)	0.73	14	98.5 (21.2)	9	174.3 (80.4)	0.97														
4h 100u	15	22.9 (4.4)	11	22.6 (9.4)	0.3	15	23.8 (7.5)	11	29.9 (13.8)	0.96	15	104.5 (28.1)	11	126.8 (64.7)	0.28														
4h 1000u	15	29.5 (6.5)	10	22.5 (8.2)	0.28	15	27.4 (8)	10	49.2 (27.2)	0.56	15	121.1 (25.3)	10	202.1 (68)	0.68														
EVR					No EVR					EVR					No EVR					EVR					No EVR				
n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value					
Unstim	23	2.5 (0.6)	2	0.8 (0.2)	N/A	23	2.8 (0.9)	2	0.4 (0.3)	N/A	23	11.6 (3.1)	2	1 (0.7)	N/A														
2h 100u	19	11.8 (3.6)	2	12 (4.3)	N/A	19	14.7 (7.5)	2	1.2 (0.6)	N/A	19	79 (39)	2	34.5 (2.8)	N/A														
2h 1000u	20	16 (3)	2	27.9 (14.6)	N/A	20	21.6 (7.3)	2	3.5 (1.5)	N/A	20	119.4 (34.3)	2	239.7 (226.6)	N/A														
4h 100u	23	21.4 (4.3)	2	43.9 (41.7)	N/A	23	28.4 (7.9)	2	13.2 (13.2)	N/A	23	118.2 (34.7)	2	93.4 (85.2)	N/A														
4h 1000u	22	26.9 (5.5)	2	27.7 (23.7)	N/A	22	38.3 (13.3)	2	9.9 (9.5)	N/A	22	145.9 (32.9)	2	224.6 (213.4)	N/A														
EOT PCR -ve					EOT PCR +ve					EOT PCR -ve					EOT PCR +ve					EOT PCR -ve					EOT PCR +ve				
n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value					
Unstim	21	2.2 (0.5)	4	3.2 (1.8)	0.39	21	2.7 (1)	4	2.1 (0.7)	0.48	21	11.5 (3.5)	4	6.6 (3)	0.97														
2h 100u	17	9.1 (2.5)	4	23.7 (13.2)	0.17	17	6.6 (1.8)	4	42.5 (35.1)	0.28	17	43.6 (12.3)	4	211.7 (181)	0.35														
2h 1000u	18	16.7 (3.3)	4	23.6 (7.2)	0.33	18	11.5 (2.5)	4	58.2 (31.4)	0.47	18	109.2 (28)	4	225.6 (155.8)	0.70														
4h 100u	21	21 (4.8)	4	29.6 (17.4)	0.85	21	21.8 (5.6)	4	56 (35.6)	0.63	21	93.5 (22.8)	4	235.7 (168.2)	0.68														
4h 1000u	20	24.9 (5.6)	4	37.3 (14.9)	0.42	20	21.4 (6.2)	4	108.8 (59.6)	0.37	20	128.6 (28.1)	4	272 (139.1)	0.33														
SVR					No SVR					SVR					No SVR					SVR					No SVR				
n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value					
Unstim	15	2.1 (0.5)	8	2 (1)	0.87	15	2.2 (1)	8	2 (0.6)	0.63	15	11.4 (4.4)	8	8.1 (3.2)	0.92														
2h 100u	12	10.4 (3.3)	8	15 (7.1)	0.42	12	5.9 (2.2)	8	25 (17.5)	0.10	12	46.6 (16.7)	8	123.6 (90.5)	0.73														
2h 1000u	13	19 (4.2)	8	16.3 (4.6)	0.80	13	11.2 (3.3)	8	34.5 (17.2)	0.59	13	124.3 (37.1)	8	153.7 (78.4)	0.97														
4h 100u	15	25.4 (6.2)	8	20.6 (9.6)	0.50	15	22.6 (7.3)	8	35.7 (18.7)	0.87	15	104.2 (29.8)	8	139 (86.3)	0.97														
4h 1000u	15	29.1 (6.9)	7	29.5 (9.6)	0.94	15	23.9 (8)	7	70.6 (36.8)	0.53	15	126 (34)	7	226.4 (81.2)	0.18														

Table 5.16. Summary of pre-treatment ISG expression in PBMCs from genotype non-1 infected patients

Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with genotype non-1, analysed with respect to patients who achieved RVR, EVR, EOT and SVR and patients who did not. Mann-Whitney U tests were used to analyse statistical significant differences.

5.3.14 Genotype 1 infected patients who achieve SVR have significantly higher ISG expression pre-treatment

Genotype 1 infected patients have the poorest rate of response to IFN- α treatment, so it would be useful to determine which patients are unlikely to respond before commencing therapy. Therefore, we were particularly interested in correlating pre-treatment ISG expression data in these patients and overall SVR. Endogenous levels of PKR and MxA were significantly higher in the PBMCs of pre-treatment patients who went on to achieve SVR than those who did not ($p<0.05$), with OAS also being higher but not significantly so (Fig 5.12 and Table 5.17). Stimulation for 2h with either 100IU or 1000IU IFN- α resulted in higher levels of PKR and OAS expression in patients with SVR, although this did not reach significance ($p=0.08$). However, all stimulations with IFN- α for 2h resulted in consistently higher levels of MxA expression in PBMCs from patients achieving SVR than those who did not achieve SVR ($p<0.05$ for 2h stimulation with 100IU and $p<0.01$ for 2h stimulation with 1000IU). For IFN- α stimulations for 4h, expression of PKR, OAS and MxA were all significantly higher in those who went on to achieve SVR. In those with SVR, stimulation with both concentrations of IFN- α for 4h resulted in significantly more PKR expression ($p<0.05$), as was OAS expression after 4h stimulation with 100IU. 4h stimulation with 1000IU IFN- α stimulation resulted in highly significant increases of OAS expression in those with SVR ($p<0.01$) whereas both 4h time points resulted in highly significant increases of MxA in the same patients ($P<0.01$). A summary of pre-treatment ISG expression in PBMCs from genotype 1 infected patients is given in table 5.18.

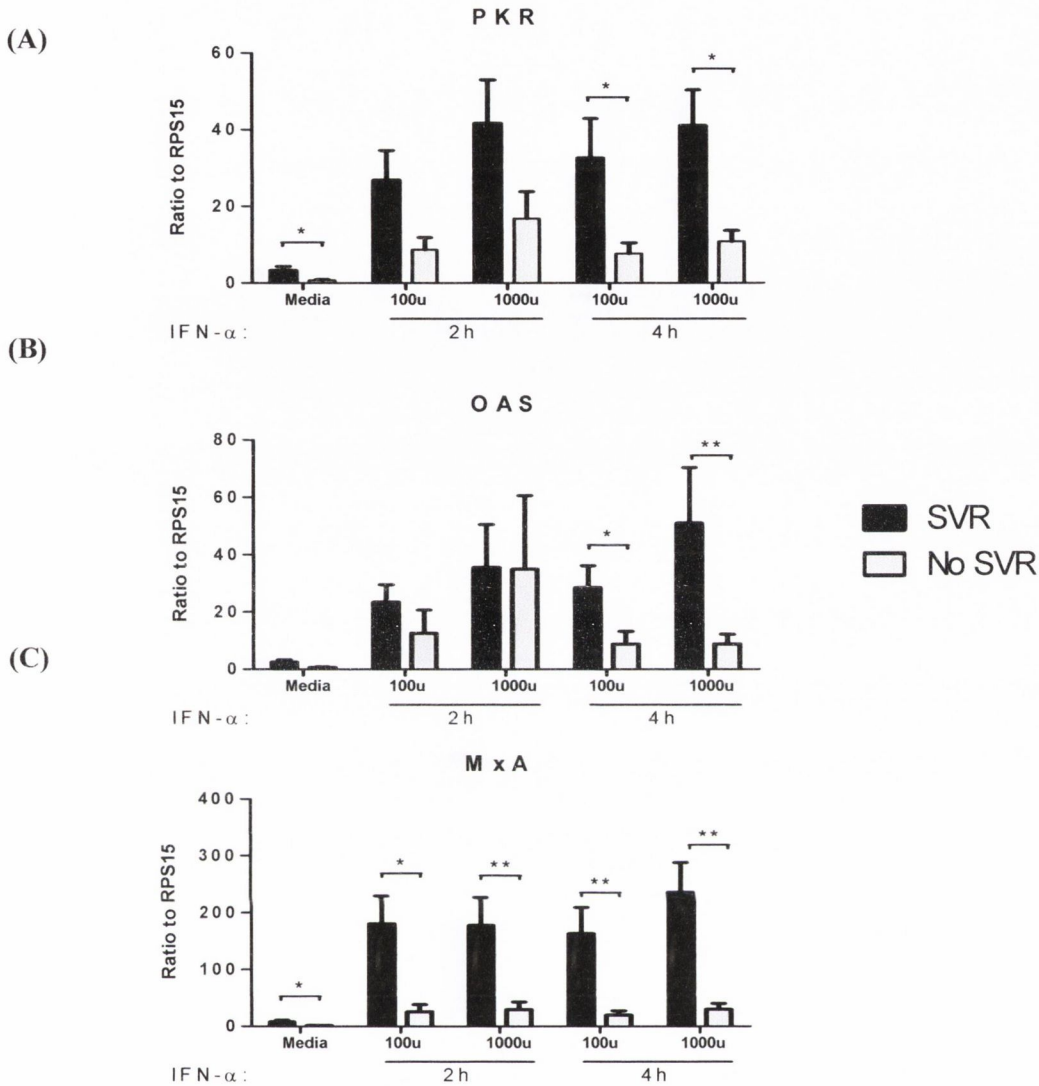


Figure 5.12. Genotype 1 patients who achieve SVR have significantly higher pre-treatment ISG expression than patients who fail to achieve SVR

PBMCs from genotype 1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved SVR and those who did not. Control media stimulated SVR and no SVR, n=8; 2h and 4h 100IU SVR and no SVR, n=5; 2h 1000IU SVR and no SVR, n=7; 4h 1000IU SVR and no SVR, n=8. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.

Genotype 1			SVR			No SVR			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		8	3.3	1.1	8	0.7	0.1	<0.05	Yes
	2h IFN-α	100IU	5	26.9	7.7	6	8.7	3.1	0.08	
		1000IU	7	41.7	11.3	6	16.8	7	0.14	
	4h IFN-α	100IU	5	32.6	10.3	7	7.7	2.8	<0.05	Yes
		1000IU	8	41.1	9.2	8	10.8	2.8	<0.05	Yes
OAS	Unstimulated		8	2.6	0.8	8	0.7	0.2	0.19	
	2h IFN-α	100IU	5	23.5	6.1	6	12.7	8	0.33	
		1000IU	7	35.6	14	6	34	25.6	0.45	
	4h IFN-α	100IU	5	28.6	7.6	7	8.8	4.5	<0.05	Yes
		1000IU	8	51	19.3	8	8.8	3.4	<0.01	Yes
MxA	Unstimulated		8	8.1	3.2	8	1.3	0.5	<0.05	Yes
	2h IFN-α	100IU	5	180.8	48.5	6	25.9	12.3	<0.05	Yes
		1000IU	7	177.8	48.8	6	29.6	13.3	<0.01	Yes
	4h IFN-α	100IU	5	163.3	45.8	7	19.7	7.9	<0.01	Yes
		1000IU	8	235.4	52.5	8	29.7	10.6	<0.01	Yes

Table 5.17. Summary of ISG expression and SVR: genotype 1
 Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with genotype 1, analysed with respect to patients who achieved SVR and those who did not.

PKR										OAS										MxA									
RVR					No RVR					RVR					No RVR					RVR					No RVR				
	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value				
Unstim	6	3.2 (1.4)	11	1.3 (0.5)	0.29	6	2.6 (0.8)	11	1 (0.5)	0.031	6	6.9 (3.5)	11	3.3 (1.8)	0.39														
2h 100IU	3	19.4 (10.4)	8	16.1 (5.5)	0.92	3	15.2 (7.9)	8	18.5 (6.8)	0.92	3	72.4 (60.3)	8	105.3 (40.7)	0.92														
2h 1000IU	5	42.3 (13.9)	8	22.6 (8.2)	0.22	5	44.1 (19.4)	8	29.8 (19.3)	0.13	5	104.2 (44.6)	8	112.6 (49.4)	0.83														
4h 100IU	3	29.6 (17)	10	15.6 (4)	0.57	3	20 (9)	10	14.7 (5.4)	0.69	3	69.5 (42.6)	10	77.6 (32.6)	0.57														
4h 1000IU	6	36.3 (11.4)	11	18.5 (6.1)	0.12	6	55.6 (25.9)	11	13.3 (4.7)	0.031	6	167.3 (77.5)	11	103 (36.5)	0.39														
EVR					No EVR					EVR					No EVR					EVR					No EVR				
	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value				
Unstim	13	2.4 (0.7)	3	0.5 (0.2)	0.1	13	1.8 (0.6)	3	0.8 (0.4)	0.5	13	5.3 (2.2)	3	2.4 (1.1)	1														
2h 100IU	9	20.3 (5.1)	2	2 (0.5)	N/A	9	21.1 (5.8)	2	1.6 (1)	N/A	9	115.2 (37.1)	2	11.3 (8.4)	N/A														
2h 1000IU	11	32.8 (8.5)	2	15.8 (13.3)	N/A	11	39.4 (15.8)	2	12.8 (12.2)	N/A	11	121.4 (38.5)	2	43.3 (38.9)	N/A														
4h 100IU	9	22.7 (7)	3	4.2 (1.3)	0.15	9	22.1 (5.5)	3	1.7 (0.8)	0.06	9	95.4 (36.2)	3	32.2 (14.4)	0.6														
4h 1000IU	13	30.9 (6.8)	3	4.4 (2.8)	0.016	13	35 (12.9)	3	3.7 (3)	0.031	13	157.1 (42.8)	3	26.2 (16.8)	0.14														
EOT PCR -ve					EOT PCR +ve					EOT PCR -ve					EOT PCR +ve					EOT PCR -ve					EOT PCR +ve				
	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value				
Unstim	11	2.6 (0.8)	5	0.7 (0.2)	0.17	11	2 (0.6)	5	0.8 (0.2)	0.57	11	6.2 (2.5)	5	1.5 (0.8)	0.14														
2h 100IU	7	20.9 (6.6)	4	10.1 (4.7)	0.41	7	17.8 (5.6)	4	17.1 (11)	0.79	7	147.3 (39.9)	4	7 (4.3)	0.006														
2h 1000IU	9	33.1 (10.3)	4	23.8 (8.6)	0.83	9	27 (12.5)	4	51.8 (36.7)	0.6	9	146.8 (42.7)	4	25.1 (19)	0.020														
4h 100IU	7	24.5 (8.8)	5	9.1 (3.8)	0.43	7	21 (7.2)	5	11.5 (5)	0.34	7	121.7 (41.5)	5	20.5 (10.7)	0.07														
4h 1000IU	11	33.3 (7.8)	5	9.8 (4.1)	0.031	11	39.4 (15)	5	9.1 (5.4)	0.041	11	185 (45.7)	5	16 (10.8)	0.007														
SVR					No SVR					SVR					No SVR					SVR					No SVR				
	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value	n	Mean (SEM)	n	Mean (SEM)	P value				
Unstim	8	3.3 (1.1)	8	0.7 (0.1)	0.028	8	2.6 (0.8)	8	0.7 (0.2)	0.19	8	8.1 (3.2)	8	1.3 (0.5)	0.021														
2h 100IU	5	26.9 (7.7)	6	8.7 (3.1)	0.08	5	23.5 (6.1)	6	12.7 (8)	0.33	5	180.8 (48.5)	6	25.9 (12.3)	0.017														
2h 1000IU	7	41.7 (11.3)	6	16.8 (7)	0.14	7	35.6 (14)	6	34 (25.6)	0.45	7	177.8 (48.8)	6	29.6 (13.3)	0.005														
4h 100IU	5	32.6 (10.3)	7	7.7 (2.8)	0.030	5	28.6 (7.6)	7	8.8 (4.5)	0.030	5	163.3 (45.8)	7	19.7 (7.9)	0.005														
4h 1000IU	8	41.1 (9.2)	8	10.8 (2.8)	0.015	8	51 (19.3)	8	8.8 (3.4)	0.002	8	235.4 (52.5)	8	29.7 (10.6)	0.002														

Table 5.18. Summary of pre-treatment ISG expression in PBMCs from genotype 1 infected patients

Summary of mean fold changes for *in vitro* ISG expression in pre-treatment patients, infected with genotype 1, analysed with respect to patients who achieved RVR, EVR, EOT and SVR and patients who did not. Mann-Whitney U tests were used to analyse statistical significant differences.

5.3.15 Genotype 1 infected patients who will respond to IFN- α therapy can be identified pre-treatment through analysis of PBMC responsiveness to IFN- α stimulation

To further assess the predictive power of measuring ISG expression *in vitro* in PBMCs to identify patients who will have an SVR, the ISG expression in genotype 1 patients following 4h stimulation with 1000IU IFN- α , the most significant time point was analysed (Fig. 5.13). To calculate the predictive power of ISG expression, we used receiver operating curve (ROC) analysis, which plots a curve of the true positive rate (the sensitivity) against the false positive rate (1-specificity). From this, the area under the curve (AUC) can be calculated, which measures the ability of the test to correctly discriminate between SVR and no SVR. A perfect test would have an area of one; tests with areas between 0.9 and 1 are considered excellent, and 0.8-0.9 is considered a good measure. For PKR, expression after IFN- α stimulation resulted in significantly higher gene expression in those with SVR (Fig. 5.13) and the AUC was 0.86 (Table 5.19). Gene expression greater than 12.2 had a 100% PPV and a 50% NPV for achieving SVR (Table 5.19), with a likelihood ratio of achieving SVR with gene expression over 12.2 of 2.67. PKR gene expression of less than 27.56 had a 100% PPV and 62.5% NPV of not achieving SVR (Table 5.20) with a likelihood ratio of not achieving SVR of 2.67. For OAS, expression after IFN- α stimulation resulted in highly significantly higher gene expression in those with SVR (Fig. 5.13) and the AUC was 0.94 (Table 5.19). Gene expression greater than 9.68 had a 100% PPV and a 25% NPV for achieving SVR (Table 5.19), with a likelihood ratio of achieving SVR with gene expression over 9.68 of 2.67. OAS gene expression of less than 31.92 had a 100% PPV and 62.5% NPV of not achieving SVR (Table 5.20), with the odds of not achieving SVR being 2.67. For MxA, expression after IFN- α stimulation resulted in highly significantly higher gene expression in those with SVR (Fig. 5.13) and the AUC was 0.94 (Table 5.19). Gene expression greater than 84.74 had a 100% PPV and an 87.5% NPV for achieving SVR (Table 5.17A), with a likelihood of achieving SVR of 7. PKR gene expression of less than 91.72 had a 100% PPV and an 87.5% NPV of not achieving SVR (Table 5.20), with not achieving SVR 8 times more likely if gene expression was less than 91.72.

The AUC values for PKR, OAS and MxA calculated deviated significantly from an imperfect AUC value of 0.5 ($p<0.01$ for all).

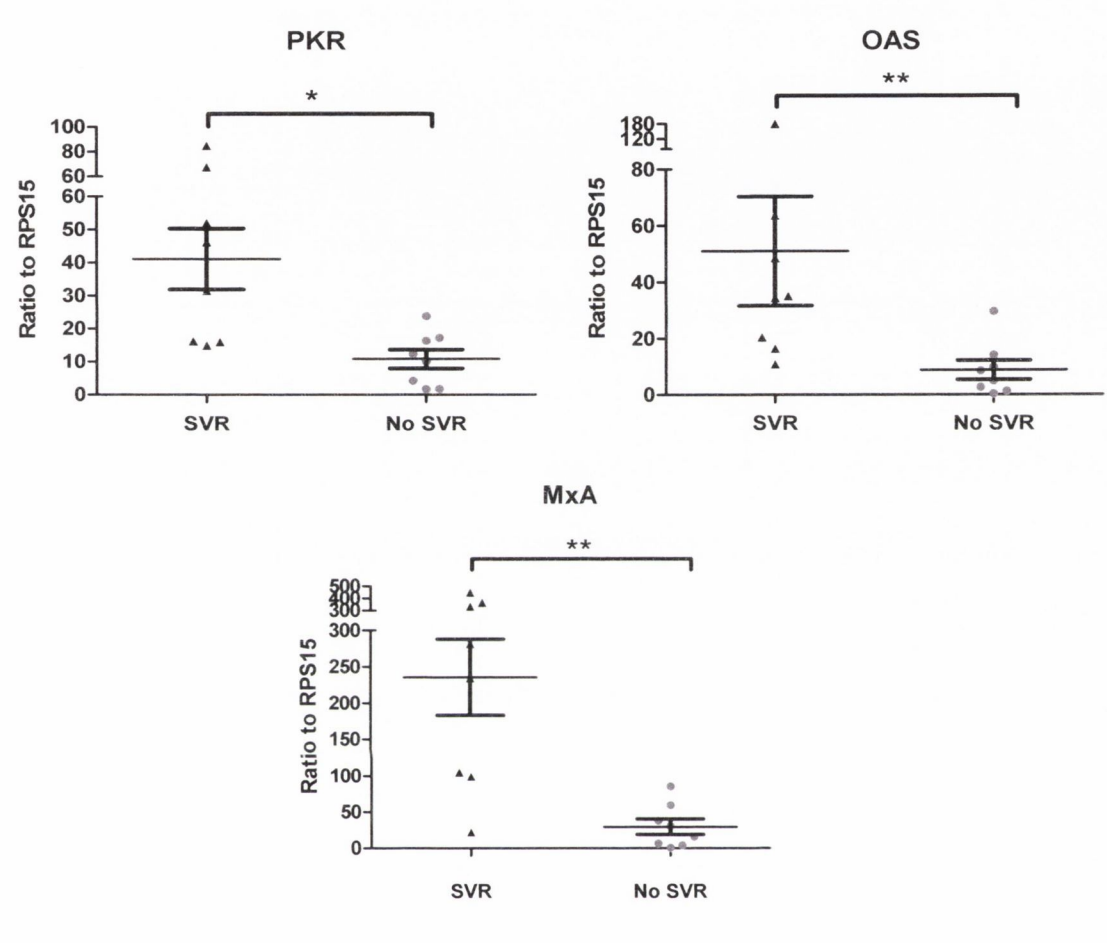


Figure 5.13. Patients with SVR have significant induction of ISGs pre-treatment

PBMCs from genotype 1 infected HCV patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with 1000IU of IFN- α for 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene expression was segregated according to those who achieved SVR and those who did not. N=8 for both SVR and no SVR. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point.* $p<0.05$; ** $p<0.01$.

Gene	AUC (95% CI)	P value	Cut-off value	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV %	NPV %	Likelihood Ratio
PKR	0.86 (0.67 - 1.1)	0.0002	>12.2	100 (63.1 - 100)	62.5 (24.5 - 91.5)	100	50	2.67
OAS	0.94 (0.82 - 1.1)	0.003	>9.68	100 (63 - 100)	75 (34.9 - 96.8)	100	25	4
MxA	0.94 (0.81 - 1.1)	0.003	>84.74	87.5 (47 - 100)	100 (63 - 100)	100	87.5	7

Table 5.19. Cut-off gene expression values for predictive values for achieving SVR

Gene	AUC (95% CI)	Model P value	Cut-off value	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV %	NPV %	Likelihood Ratio
PKR	0.86 (0.67 - 1.1)	0.0002	<27.56	100 (63.1 - 100)	62.5 (24.5 - 91.5)	100	62.5	2.67
OAS	0.94 (0.82 - 1.1)	0.003	<31.92	100 (63 - 100)	62.5 (24.5 - 91.5)	100	62.5	2.67
MxA	0.94 (0.82 - 1.1)	0.003	<91.72	100 (63 - 100)	87.5 (47 - 100)	100	87.5	8

Table 5.20. Cut-off gene expression values for predictive values for not achieving SVR

Summary of AUC analysis, optimal gene cut-off expression, sensitivity, specificity and predictive values for ISG expression in PBMCs from patients before IFN- α therapy commencement who ultimately achieve SVR.

AUC: area under the curve, CI: confidence intervals, PPV: positive predictive value, NPV: negative predictive value

5.3.16 Patients of all genotypes who relapse have low *in vitro* ISG expression pre-treatment

Overall response to therapy is determined 6 months after treatment because viral breakthrough (i.e. becoming PCR +ve) can occur within this time, despite the patient being PCR –ve at the end of treatment. In our cohort, the viral relapse rate was 23%, 25% for those infected with genotype 1 and 21% for those infected with other genotypes. We wanted to determine if there was any correlation between IFN- α induced ISG expression and relapse to therapy. We found that in patients infected with all genotypes of HCV, endogenous ISG expression and IFN- α induced ISG expression was decreased pre-treatment in the patients who went on to relapse after therapy (Fig. 5.14 and Table 5.21). The correlation between ISG expression and patients who were PCR +ve within the 6 months after treatment was most pronounced for PKR, which was significantly less in relapsers, compared to patients who achieved SVR, following stimulation with 1000IU IFN- α for 2h ($p<0.05$). This was similar following stimulation with 100IU IFN- α for 4h, although not significant ($p=0.06$). OAS and MxA expression was also less in the relapsers, although this was not significantly so, possibly due to the low patient numbers involved in the analysis.

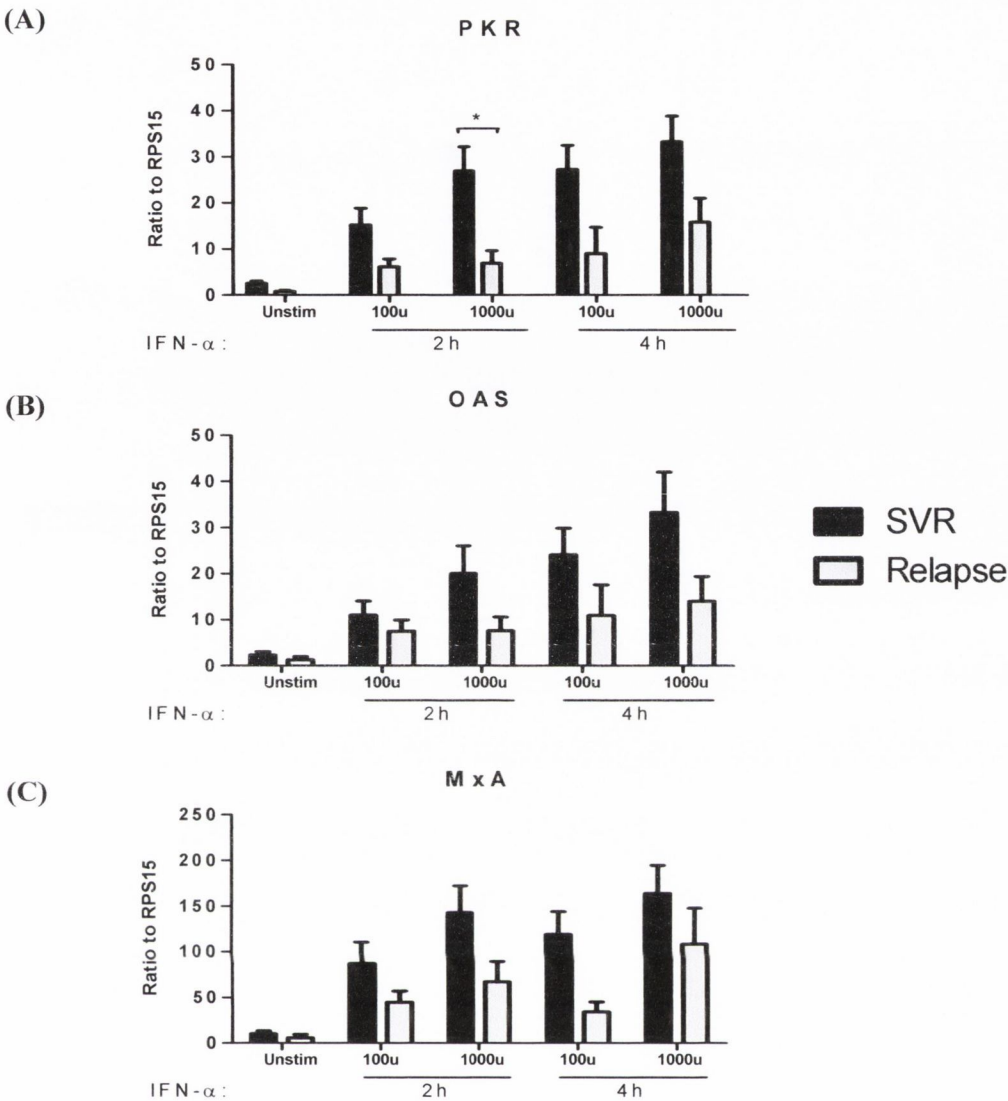


Figure 5.14. Patients (all genotypes) who relapse after treatment have lower ISG expression pre-treatment

PBMCs from HCV patients (all genotypes) were isolated prior to the commencement of IFN-α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN-α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene induction was segregated according to those who relapsed (became PCR +ve within 6 months of treatment) and those who did not. No relapse: control, n=23; 2h 100IU, n=17; 2h 1000IU & 4h 100IU, n=20; 4h 1000IU, n=23; relapse: control, n=7; 2h 100IU & 1000IU, 4h 100IU & 1000IU, n=6. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point. *p<0.05; **p<0.01.

All genotypes			No relapse			Relapse			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		23	2.5	0.5	7	0.8	0.2	0.09	
	2h IFN-α	100IU	17	15.2	3.7	6	6.2	1.7	0.46	
		1000IU	20	26.9	5.2	6	6.9	2.7	<0.05	Yes
	4h IFN-α	100IU	20	27.2	5.2	6	9	5.7	0.06	
		1000IU	23	33.3	5.5	6	15.8	5.2	0.15	
OAS	Unstimulated		23	2.3	0.7	7	1.3	0.7	0.52	
	2h IFN-α	100IU	17	11	3	6	7.5	2.4	0.97	
		1000IU	20	20	6	6	7.6	3	0.23	
	4h IFN-α	100IU	20	24.1	5.7	6	10.9	6.7	0.31	
		1000IU	23	33.3	8.7	6	14	5.4	0.25	
MxA	Unstimulated		23	10.3	3.1	7	5	3.7	0.38	
	2h IFN-α	100IU	17	87.5	23.1	6	44.9	12.2	0.70	
		1000IU	20	143	29.4	6	67.4	22.3	0.24	
	4h IFN-α	100IU	20	119	25.2	6	34.2	11	0.12	
		1000IU	23	164	30.6	6	108.3	39.6	0.63	

Table 5.21. Summary of ISG expression and relapse: all genotypes
 Summary of mean fold changes for *in vitro* ISG induction in pre-treatment patients, infected with all viral genotypes, analysed with respect to patients who achieved SVR and those who relapsed after having an EOT response.

5.3.17 Genotype non-1 infected patients who relapse have low ISG expression pre-treatment

21% of genotype non-1 infected patients relapsed, despite being PCR –ve for HCV when treatment was completed. 50% of these genotype non-1 infected patients had previously achieved an RVR and an EVR and were therefore PCR –ve throughout therapy yet had viral breakthrough once therapy was stopped. Although ISG induction in this group of patients showed no correlation with treatment response at any stage of therapy, we wanted to assess if ISG induction could be associated with treatment relapse. Endogenous expression of PKR, OAS and MxA in unstimulated cells was less in the patients who relapsed following therapy compared to those who did not (Fig. 5.15 and Table 5.22). Induction of PKR was much lower in these patients following IFN- α , regardless of the concentration of IFN- α or the time point examined. OAS and MxA induction were also moderately reduced. Overall, relapsers infected with genotypes other than genotype 1 of HCV showed a general reduction in ISG expression pre-treatment compared to patients who remained PCR –ve, but this never reached significance.

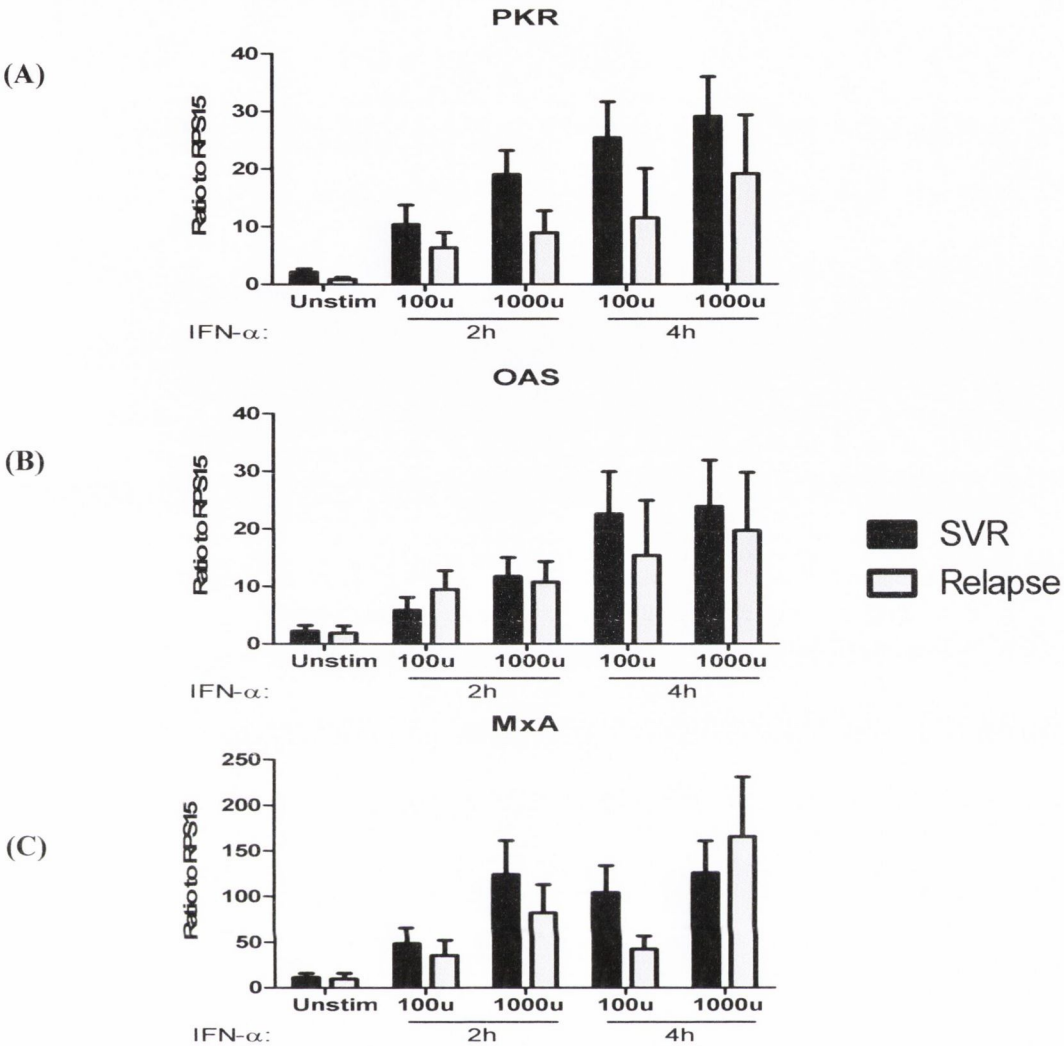


Figure 5.15. There is no significant difference in ISG expression in patients infected with genotype non-1 who relapse and those who achieve SVR

PBMCs from genotype non-1 infected patients were isolated prior to the commencement of IFN-α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN-α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene induction was segregated according to those who relapsed and those who did not. No relapse: control, n=15; 2h 100IU, n=12; 2h 1000IU, n=13; 4h 100IU & 1000IU, n=15; relapse: control, 2h 100IU & 1000IU & 4h 100IU, n=4, 4h 1000u, n=3. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point. *p<0.05; **p<0.01.

Genotype non-1			No relapse			Relapse			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		15	2.1	0.5	4	0.9	0.4	0.34	
	2h IFN-α	100IU	12	10.4	3.3	4	6.3	2.6	0.86	
		1000IU	13	19	4.2	4	8.9	3.8	0.28	
	4h IFN-α	100IU	15	25.4	6.2	4	11.5	8.6	0.34	
		1000IU	15	29.1	6.9	3	19.2	10.2	0.64	
OAS	Unstimulated		15	2.2	1.9	4	1	1.3	0.96	
	2h IFN-α	100IU	12	5.9	9.5	4	2.2	3.3	0.25	
		1000IU	13	22.6	10.7	4	7.3	3.6	0.65	
	4h IFN-α	100IU	15	22.6	15.4	4	7.3	9.6	0.88	
		1000IU	15	23.9	19.7	3	8	10.1	1	
MxA	Unstimulated		15	11.4	4.4	4	9.6	6.2	0.96	
	2h IFN-α	100IU	12	48.6	16.7	4	35.5	16.7	0.95	
		1000IU	13	124.3	37.1	4	81.9	31.1	0.87	
	4h IFN-α	100IU	15	104.2	29.8	4	42.4	14.5	0.58	
		1000IU	15	126	34	3	165.7	65.3	0.34	

Table 5.22. Summary of ISG expression and relapse: genotype non-1
 Summary of mean fold changes for *in vitro* ISG induction in pre-treatment patients, infected with all viral genotypes except genotype 1, analysed with respect to patients who achieved SVR and those who relapsed after having an EOT response.

5.3.18 Genotype 1 infected patients who relapse have low ISG induction pre-treatment.

25% of patients examined who were infected with genotype 1 were PCR –ve at the end of 48 weeks of IFN- α /ribavirin therapy, yet became PCR +ve within the 6 months after treatment and therefore relapsed. Not all genotype 1 infected relapsers had an RVR but all had an EVR. Endogenous expression of PKR, OAS and MxA was not significantly different in the PBMCs of patients pre-treatment who subsequently went on to relapse after therapy, compared to patients who achieved SVR (Fig. 5.16 and Table 5.23). Unfortunately, for stimulation for 2h with 100IU and 1000IU IFN- α , or stimulation with 100IU IFN- α for 4h, the number of patients who relapsed was too low to analyse (n=2); however, the data for these 2 relapser patients showed lower ISG expression in these patients than in patients who remained PCR –ve after treatment. For stimulation with 1000IU IFN- α for 4h, induction of PKR, OAS and MxA were all lower in patients who relapsed compared to those who did not. The reduction in OAS expression in these patients was significantly less ($p<0.05$) but not for PKR and MxA.

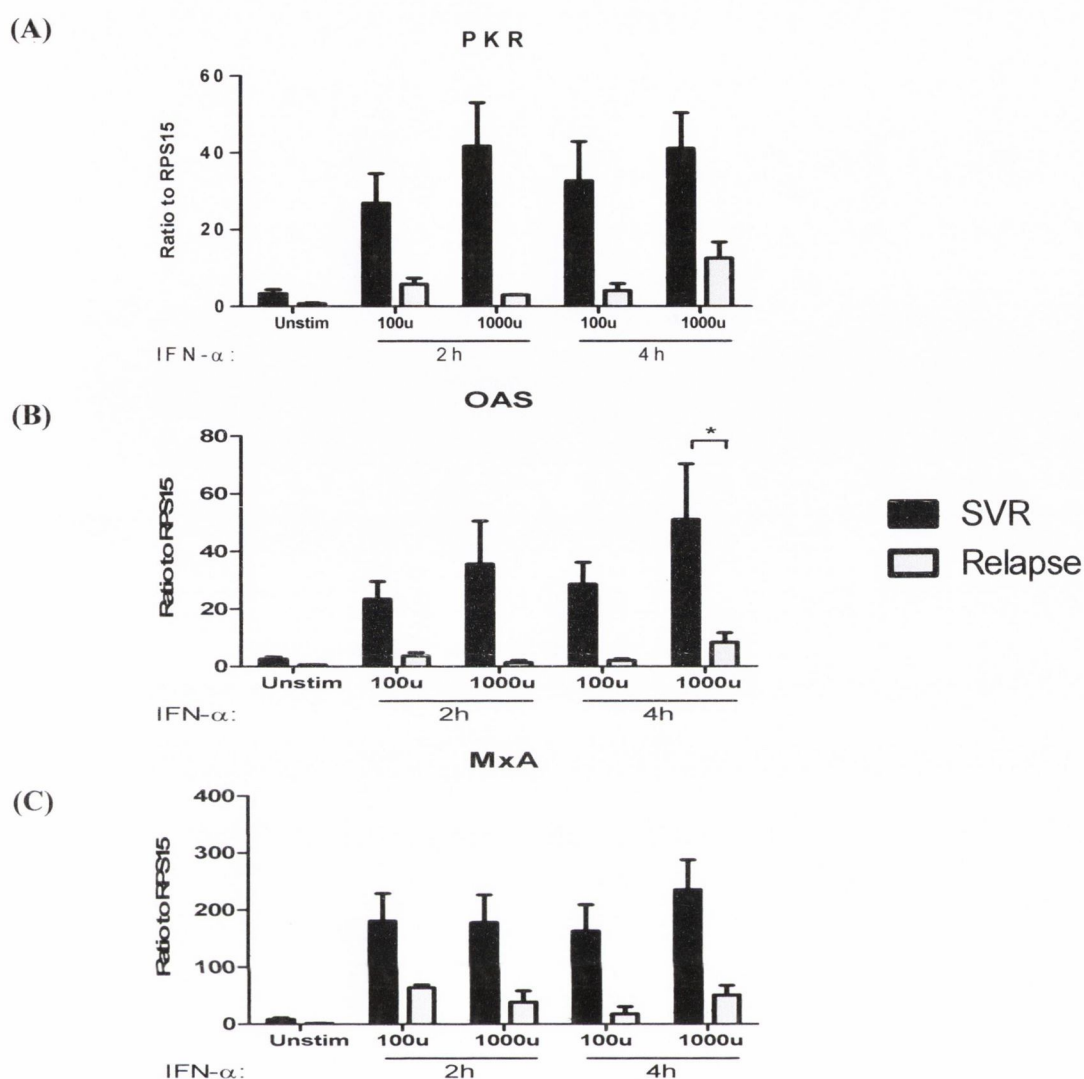


Figure 5.16. Genotype 1 infected patients who relapse have lower ISG expression pre-treatment

PBMCs from genotype 1 infected patients were isolated prior to the commencement of IFN- α treatment and stimulated *in vitro* with either 100IU or 1000IU of IFN- α for 2 or 4h. qRT-PCR was used to measure the mRNA for PKR, OAS and MxA, which were normalised relative to the internal housekeeper RPS15. Gene induction was segregated according to those who relapsed (became PCR +ve within 6 months of treatment) and those who did not. No relapse: control, n=8; 2h 100IU, n=5; 2h 1000IU, n=7; 4h 100IU, n=5; 4h 1000IU, n=8; relapse: control, n=3; 2h 100IU & 1000IU & 4h 100IU, n=2; 4h 1000IU, n=3. Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups at each time point. *p<0.05; **p<0.01.

Genotype 1			No relapse			Relapse			P value	Significant
			N	Mean	SEM	N	Mean	SEM		
PKR	Unstimulated		8	3.3	1.1	3	0.7	0.3	0.13	
	2h IFN-α	100IU	5	26.9	7.7	2	5.8	1.5	N/A	
		1000IU	7	41.6	11.3	2	2	0.1	N/A	
	4h IFN-α	100IU	5	32.6	10.3	2	4.1	1.8	N/A	
		1000IU	8	41.1	9.2	3	12.5	4.2	0.28	
OAS	Unstimulated		8	2.6	0.8	3	0.5	0.1	0.38	
	2h IFN-α	100IU	5	23.5	6.1	2	3.7	1.	N/A	
		1000IU	7	35.6	14	2	1.3	0.8	N/A	
	4h IFN-α	100IU	5	28.6	7.6	2	2.1	0.5	N/A	
		1000IU	8	51	19.3	3	8.3	3.3	<0.05	Yes
MxA	Unstimulated		8	8.1	3.2	3	1.1	0.4	0.08	
	2h IFN-α	100IU	5	180.8	48.5	2	63.7	5.6	N/A	
		1000IU	7	177.8	48.8	2	38.4	19.7	N/A	
	4h IFN-α	100IU	5	163.3	45.8	2	17.8	12.6	N/A	
		1000IU	8	235.4	52.5	3	50.9	17.1	0.08	

Table 5.23. Summary of ISG expression and relapse: genotype 1
 Summary of mean fold changes for *in vitro* ISG induction in pre-treatment patients, infected with genotype 1, analysed with respect to patients who achieved SVR and those who relapsed after having an EOT response.

5.3.19 CXCL10 expression pre-treatment is similar in genotype 1 infected patients who have an SVR and those who do not

We previously found that patients who had been treated with IFN- α and failed to respond had significantly higher endogenous levels of the chemokine CXCL10, when compared to patients who had also undergone therapy but responded (Fig. 4.14). Therefore, as this retrospective analysis revealed significant differences in CXCL10 in responders and non-responders after treatment, we wanted to assess CXCL10 mRNA in patients pre-treatment. We analysed CXCL10 mRNA levels in the pre-treatment PBMCs from patients infected with genotype 1 (Fig. 5.17). We found that there was no significant difference in endogenous CXCL10 mRNA expression in the PBMCs between patients who had an SVR and those who did not. Gene induction following IFN- α stimulation also showed no difference in the level of expression of CXCL10 mRNA between patients who achieved SVR and those who did not.

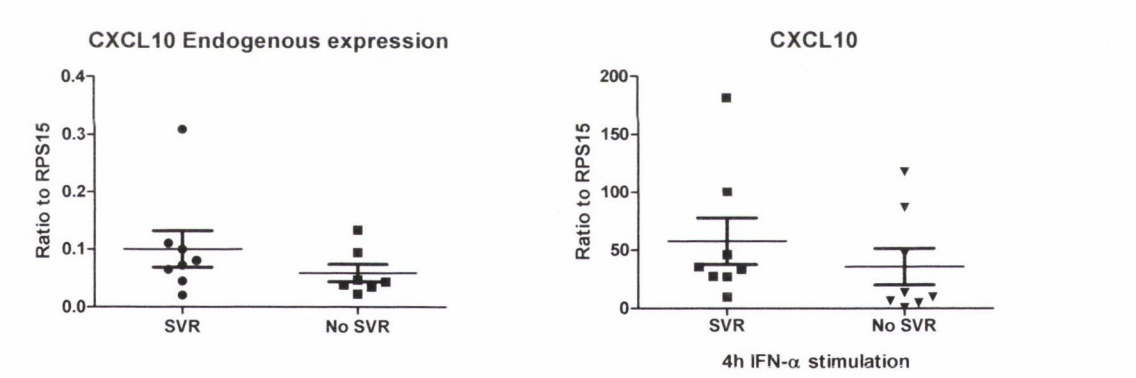


Figure 5.17. CXCL10 mRNA expression is similar in genotype 1 infected patients who achieve SVR and those who do not

PBMCs were isolated from genotype 1 infected patients prior to the commencement of IFN- α therapy and stimulated with 1000IU IFN- α for 4h. CXCL10 mRNA was quantified using qRT-PCR. All gene expression values are expressed as a ratio to the internal housekeeping control RPS15. Gene expression was segregated according to patients who subsequently achieved SVR or did not at the end of treatment.

5.4 Discussion

Our earlier studies revealed differences in responsiveness of immune cells to IFN- α stimulation between patients who had successfully cleared the virus following therapeutic IFN- α and ribavirin compared with patients who had failed to respond. Patients who did not respond had significantly lower IFN- α induced anti-viral gene expression *in vitro*. Therefore we hypothesised that by analysing the *in vitro* response of PBMCs to IFN- α prior to commencing treatment, we could predict who would respond to therapy and those who would not respond.

The overall SVR rate in our patient cohort was 62%; 50% for genotype 1 infected patients and 65% for those infected with other genotypes. This SVR rate is lower than what has been reported previously, particularly for the patients infected with genotype non-1 [2]. This is most likely due to the low patient number in the study. 90% of patients who achieve an RVR will achieve an SVR, as this difference was no significant between our response rates and another study of response rates in patients attending SVUH (Fisher's exact T test, $p=0.46$) [226]. We found that 81% of our patients who achieved RVR went on to have SVR, similar to what had been previously reported in a cohort of patients treated in the Hepatology Unit in SVUH [4]. We found that 63% of patients with no RVR had no SVR, whereas the previous study found that no RVR predicted 87.5% of those who would not respond. This may be due to the lower patient number in our study ($n=45$ vs $n=190$, respectively). Most patients in our cohort achieved EVR (81%); again for genotype 1 infected patients the EVR rate was slightly lower than in patients infected with other genotypes (80% vs 82%, respectively), as would be expected. 81% of patients were PCR -ve at the end of treatment, but only 62% achieved an SVR. This meant that 23% of patients had viral breakthrough within the 6 months after treatment and were therefore considered relapsers. Details on the rates of relapse after successfully achieving an EOT response is limited, however a Spanish study in 2009 found similar relapse rates within their patient cohort (22%) [246].

It has already been demonstrated using microarray analysis that the *in vitro* stimulation of PBMCs with IFN- α reflects ISG upregulation *in vivo* in HCV patients

[4]. In fact, patients who fail to respond to IFN- α have a decreased global transcriptional response to *in vitro* IFN- α stimulation pre-treatment in their PBMCs [244]. High endogenous expression of ISGs in the liver is strong predictors of non-response to therapy [245]. However, liver biopsies are an invasive procedure associated with significant risks, whereas PBMCs represent a far more assessable source of patient material for assessing treatment response [231]. Therefore, as ISG expression is a potentially good marker for treatment response, our prospective study examined ISG induction in PBMCs pre-treatment using quantitative real-time PCR, to link pre-treatment response to IFN- α stimulation *in vitro* with therapeutic IFN- α response.

A useful measure of response to treatment is to monitor viral kinetics at week 4 and week 12 during therapy, with the probability of achieving SVR strongly linked to early viral clearance during therapy. We found that for all HCV genotypes, endogenous ISG expression tended to be lower pre-treatment in patients who did not achieve RVR. Analysis of stimulated PBMCs from patients infected with all genotypes of HCV revealed that expression of PKR, OAS and MxA was similar in those with RVR and those without, with the exception of PKR which was significantly higher in patients with RVR following 4h stimulation with 1000IU IFN- α . When we analysed these data according to viral genotypes, discernable differences were observed. Overall, those infected with genotype non-1 tended to have lower ISG expression following IFN- α treatment in the patients who did have an RVR and higher ISG expression in those who did not achieve RVR. However, genotype 1 infected patients who had an RVR tended to have higher pre-treatment ISG expression following *in vitro* IFN- α stimulation in their PBMCs, with only OAS being significantly higher in those with RVR following 4h stimulation with 1000IU IFN- α . Therefore, these results indicate that pre-treatment ISG induction does not predict RVR.

At week 12 of treatment, patients who have $>2 \log_{10}$ drop in viral load have achieved an EVR. Patients who achieve EVR have a 65% likelihood of achieving SVR, whereas patients who so not achieve EVR only have a 1-3% chance of responding to treatment; therefore treatment is halted at this point for all patients, regardless of viral genotype [247]. As with RVR, we analysed our pre-treatment ISG induction data with respect to the patients who achieved EVR and those who did not. Initially, we analysed all genotypes of HCV and found that ISG expression, either endogenous or following

IFN- α stimulation, tended to be higher in patients pre-treatment who then went on to have an EVR, but only differences in OAS expression reached significance. In the genotype non-1 patients, only 2 patients had an EVR, as a result, definitive conclusions about pre-treatment ISG induction and EVR cannot be made in these patients. In the genotype 1 infected patients, the EVR rate was 83% (15/18), therefore the numbers of those without EVR was low. However from this limited number, a trend did emerge for ISG expression. Those who did not have an EVR had lower endogenous expression of the 3 anti-viral genes and following IFN- α stimulation, they had lower expression of these compared to those with EVR. This was statistically significant for OAS and PKR expression, despite the small number of patients analysed.

When we examined pre-treatment ISG induction with respect to EOT response, we found similar patterns of expression as seen with RVR and EVR. Generally, patients infected with all HCV genotypes who were PCR –ve at the end of treatment had similar pre-treatment ISG expression compared to those who were PCR +ve. Similarly, patients infected with genotypes other than genotype 1 had similar ISG pre-treatment in patients with an EOT response and those without. However, there was a trend in patients who remained PCR +ve at EOT to have slightly higher ISG expression pre-treatment than those who became PCR –ve. More definitive trends emerged through the analysis of genotype 1 infected patients. Expression of PKR, OAS and MxA after stimulation with IFN- α was significantly higher in patients pre-treatment who went on to be PCR –ve at EOT.

Although the link between pre-treatment ISG expression and RVR, EVR and EOT response is of interest, ultimately it is the correlation with SVR that is of importance for predicting response to IFN- α therapy. Initially, we analysed SVR and ISG expression in PBMCs from HCV patients infected with all genotypes. Overall, PKR and MxA expression tended to be lower in patients who did not have an SVR, but this was not statistically significant. OAS expression was similar in those with SVR and those who did not achieve SVR. Pre-treatment ISG expression was similar in patients infected with genotype non-1 who achieved SVR and those who did not. Therefore, pre-treatment ISG expression could not predict SVR in patients infected with genotype non-1. In contrast, pre-treatment ISG expression in genotype 1 infected patients was a robust predictor of SVR. In genotype 1 infected patients, we found that endogenous ISG

expression in PBMCs was significantly lower pre-treatment in patients who failed to respond to therapy. This is different to what had been reported previously in the liver, as patients who fail to have an SVR have significantly higher pre-treatment hepatic ISG expression [6]. Following IFN- α stimulation for 4h, all 3 anti-viral genes were significantly higher pre-treatment in patients who achieved SVR than those who did not. Interestingly, expression of MxA mRNA showed the greatest differences between responders and non-responder despite this being the gene least associated with RVR or EVR.

The association of pre-treatment ISG expression in PBMCs from genotype 1 infected patients and achieving SVR was greatest following stimulation with 1000IU IFN- α for 4h, therefore we further analysed the predictive power of this stimulation to discriminate between responders and non-responders. Of particular importance was to determine if we could identify patients who would respond to standard IFN- α and ribavirin therapy and would therefore not require addition of a protease inhibitor. Firstly, we calculated the area under the curve (AUC) for each gene using ROC analysis. AUC measures the ability to correctly discriminate between those with SVR and those with no SVR. The null hypothesis is if you cannot distinguish between the two groups, AUC=0.5. A perfect test to accurately discriminate between two groups would result in AUC=1 [248]. We found that pre-treatment expression of all three anti-viral genes were strong predictors of over SVR (PKR, AUC=0.86; OAS, AUC=0.94; MxA, AUC=0.94). This further highlights the predictive power of studying these anti-viral genes pre-treatment.

We also calculated predictive values for achieving SVR and not achieving SVR, based on different anti-viral gene expression cut-off levels, as determined using ROC analysis. Gene expression of PKR greater than 12.2, OAS greater than 9.7 and MxA greater than 84.5 predicted SVR in 100% of patients tested. MxA had the strongest predictive power, with patients with MxA expression greater than 84.5 being 7 times more likely to achieve SVR with standard IFN- α and ribavirin, and would therefore not require treatment with new protease inhibitors. We found that gene expression cut-off values of 27.6 for PKR, 31.9 for OAS and 91.7 for MxA could predict likelihood of not achieving SVR in 100% of our patients. This was particularly stringent for MxA expression where patients with gene expression less than 91.7 were 8 times more likely

not to achieve SVR as patients who had gene expression higher than this value. Overall, pre-treatment MxA gene expression was the greatest predictor of patients who would or would not achieve SVR and using this pre-treatment expression of this gene, we accurately identified patients who would not need intervention with triple therapy and patients who would benefit from it.

Another subset of patients we were interested in analysing were those who relapsed after treatment completion; these were patients who achieved an EOT response but had viral re-occurrence in the first 6 months after completing IFN- α therapy. PBMCs are a likely extrahepatic reservoir of HCV that may be responsible for re-infection of the liver after treatment [249], therefore the pre-treatment responsiveness of PBMCs to *in vitro* IFN- α stimulation could be a potential predictor of relapse. 23% of patients within our study relapsed after therapy; 25% of genotype 1 infected patients and 21% of genotype non-1 infected patients. The patient number was quite low therefore definitive conclusions could not be made, however trends in ISG induction could be examined. We found that patients who relapsed tended to have less ISG induction pre-treatment in their PBMCs, regardless of viral genotype and this was significantly different for OAS expression. When divided up into genotype 1 and genotype non-1 infected patients, again those who relapsed had less ISG induction in their PBMCs than patient who successfully achieved an SVR. We would predict that with larger numbers in the relapse cohort, particularly for genotype 1 infected patients, there would be a significant difference in pre-treatment ISG expression in the PBMCs from responders compared to relapsers.

Our retrospective studies had found that CXCL10 mRNA was higher in patients who had failed to respond to therapy compared to patients who had responded (Fig. 4.14). CXCL10 protein has been reported to be higher in the pre-treatment serums of patients who subsequently fail to respond to IFN- α therapy [207,250]. Therefore, we wanted to evaluate the mRNA gene expression for CXCL10 pre-treatment in PBMCs from HCV patients and determine if there was any correlation with treatment outcome. We found no correlation in either endogenous gene levels, or expression after stimulation, for CXCL10 mRNA and SVR. CXCL10 protein levels were also evaluated in this patient cohort in a parallel study, with no association found between CXCL10 protein levels and treatment response (Dr. Shahzad Sarwar, SVUH, personal

communication). Therefore our mRNA expression data for CXCL10 correlates with the CXCL10 protein data for these patients, as neither could predict SVR.

In conclusion, pre-treatment ISG expression in PBMCs from HCV patients infected with genotype 1 can predict response to IFN- α treatment. Using specific gene expression data, we could identify pre-treatment genotype 1 infected patients who would respond to IFN- α and ribavirin combination therapy and patients who would need additional treatment with protease inhibitors during therapy. This type of predictive assay would result in substantial economic savings, as well as avoid the need for an additional anti-viral agent with its inherent adverse effects. RVR, EVR and EOT responses cannot be reliably predicted pre-treatment from ISG expression in PBMCs, most likely because on treatment viral kinetic analysis is not a robust predictor of SVR in this patient cohort. Pre-treatment ISG expression in PBMCs from genotype 1 infected patients can predict SVR, which is the ultimate aim of treatment as it eradicates the virus.

Chapter 6

Final Discussion

Chapter 6: Final Discussion

Since the identification of HCV in 1989, Scientists have sought to understand how HCV establishes persistent infection within the human body. Viral infection normally activates a potent anti-viral state within the body, first with the activation of innate immune defences which in turn activate specific adaptive immune responses, capable of retaining immunological memory. Although the risk of HCV infection via contaminated blood products has decreased with enhanced screening of blood, those infected with HCV through IVDA is still increasing. Also, new cohorts of infected patients with acute HCV infection are also emerging, including through sexual contact, particularly in those infected with HIV and in men who have sex with men (MSM) [251]. In addition, re-infection rates of HCV in patients who had previously cleared the virus are rising, particularly in the MSM cohort [252]. Therefore, it remains imperative that the immune response to HCV be fully elucidated, thus allowing for the development of novel therapeutics and improved treatment regimes.

Viruses often subvert innate immunity to prevent triggering of anti-viral immune responses. Type I IFNs are rapidly produced by the innate immune system following viral infection and act as critical regulators of both innate and adaptive anti-viral immune responses. IFN- α is a family of type I IFNs that signal via STAT transcription factors to promote the upregulation of 100s of ISGs [3]. These ISGs activate an array of different cellular responses including control of the cell cycle and many metabolic pathways [99]. They particularly upregulate many processes involved in anti-viral defence, including MHC processing, cytokine expression and molecules that directly target viruses for elimination [253]. Therefore, many viruses have evolved mechanisms to subvert both the upregulation of type I IFNs and inhibit their signalling in an effort to block anti-viral innate defences. This robust induction of anti-viral immunity by IFN- α has led to its development as a therapy for HCV, although therapy with IFN- α often fails to eradicate viral infection from the host, suggesting that HCV directly inhibits the action of this cytokine.

Chapter 3 of this thesis sought to investigate a specific mechanism by which HCV blocks the action of IFN- α and evades innate immune responses. Previous results

from our lab demonstrated that HCV degrades STAT3 protein in PBMCs from HCV patients in a proteasome-dependent manner. Several viruses hi-jack the host proteasome system to target STAT proteins for degradation; one such example is RSV. RSV encodes a viral E3 ligase contained a VHL-box, which mediates binding to elongin C and cul-2, thus allowing the virus to polyubiquitinate STAT2 and target it for proteasomal degradation [147]. Bioinformatic analysis identified a putative elongin C binding site within the p7 protein of HCV. Further analysis revealed the presence of an additional elongin C binding site within NS2 protein and a cul-2 binding site within E2 protein. Interestingly, these binding sites were highly conserved within genotype 1 of HCV and not other genotypes. As genotype 1 of HCV is most resistant to IFN- α therapy [2], we hypothesise this is because of its ability to degrade STAT proteins, thus blocking IFN- α signal transduction and the upregulation of ISGs essential for viral clearance. However, as the amino acid sequence of these binding sites were the same in genotype 1a and 1b, they do not explain the differences in response between these two subtypes to IFN- α therapy [66].

E3 ligases with VHL-boxes are generally single proteins capable of binding elongin C and cul-2; however, the two elongin C and the cul-2 binding site were in the HCV proteins p7, NS2 and E2 respectively. It is known that the HCV polypeptide is incompletely cleaved during viral processing and leads to the generation of E2-p7-NS2 and E2-p7 proteins [187]. Therefore, we sought to determine if E2-p7-NS2 (E2NS2) could act as a possible viral E3 ligase and degrade STAT3. We found that expression of E2NS2 could abolish STAT3 protein expression. Furthermore, similar to expression of full length HCV, E2NS2 was capable of inhibiting activation of ISRE and GAS response elements following IFN- α stimulation and could block the induction of the ISGs PKR and MxA, indicating that this region was indeed responsible for inhibiting IFN- α signalling.

Having identified a mechanism by which HCV suppresses activation of anti-viral responses to IFN- α , we hypothesised that if the elongin C and cul-2 binding sites allow HCV to act as a functional E3 ligase, then specifically targeting these sites would reverse HCV degradation of STAT proteins and restore IFN- α induced anti-viral genes. Therefore, we synthesised small peptide mimics of these viral binding sites, designed to bind elongin C and cul-2, thus preventing HCV recruitment of these proteins and

subsequent inhibition of IFN- α signalling. Treatment with the three peptides in combination restored expression of STAT3 protein and IFN- α induced anti-viral genes. These results confirm that E2NS2 is acts as an E3 ligase and these binding sites mediate recruitment of elongin C and cul-2 proteins to degrade STAT proteins (Fig. 6.2). These investigations have paved the way for future research within our lab to develop these peptides further, with the intention of designing small molecule inhibitors against these binding sites which could be used as a future therapy that would both increase responsiveness to IFN- α therapy and possibly restore host responsiveness to endogenous IFN- α .

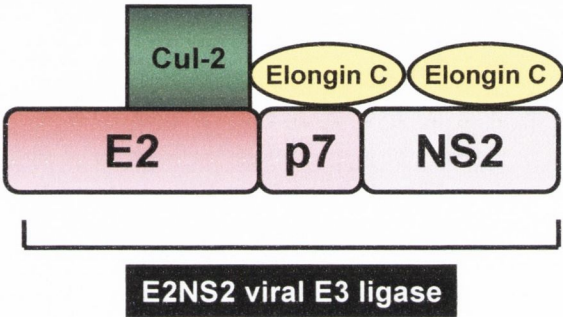


Figure 6.1. E2NS2 is a viral E3 ligase

The E2-p7-NS2 polyprotein can act as an E3 ligase to degrade STAT3 and inhibit innate immune responses. In HCV genotype 1, E2 protein contains a binding site for cul-2, p7 protein contains a binding site for elongin C and NS2 protein contains an additional binding site for elongin C. Peptides against these binding sites can restore responsiveness to IFN- α .

The interaction of HCV and the immune system involves two aspects: viral and host factors. Firstly, the impact of viral genotype is clear when response rates to IFN- α therapy are examined, as genotype 1 infected patients have a much poorer response rate to therapy than other genotypes [2]. Chapter 3 of this thesis investigated why genotype 1 was most resistant to therapy and the mechanism used by HCV to subvert the actions of IFN- α . Secondly, there is also a host immune related feature involved as not everyone infected with the same viral genotype responds to HCV infection and IFN- α therapy similarly. The importance of host genetics is demonstrated through study of a cohort of Irish women infected with anti-D contaminated with genotype 1b of HCV in the 1970s. Over 1,500 women are known to have received contaminated anti-D, of

these 704 tested antibody positive for HCV, with 390 also testing PCR +ve for the virus, indicating persistent infection within this subgroup of women [71]. That means that 314 women successfully mounted an adaptive immune response capable of clearing the virus. Even more striking are the women who received contaminated anti-D but have no immunological memory of infection, which is around 55% known to have received contaminated anti-D. This indicates that the innate immune system within these women was capable of eliminating the virus before adaptive immune responses were mounted. Analysis of the innate immune system in these women who seemingly cleared HCV infection without any induction of the adaptive immune response would possibly reveal the innate immune mechanisms crucial for viral clearance and response to IFN- α therapy and increase our overall understanding of the innate anti-viral system.

Our lab previously found that STAT1 and STAT3 expression was reduced in PBMCs from HCV patients (Fig. 4.3). As clinical IFN- α strongly upregulates ISG expression in PBMCs compared to liver [219], we postulated that this could be a possible reason for non-response to therapy and that analysis of responsiveness of PBMCs to IFN- α stimulation may predict therapeutic response. Our hypothesis was that interactions between HCV and the innate immune system, specifically the IFN- α response within PBMCs from infected patients, would have major implications for response to therapeutic IFN- α .

Initially, we sought to examine anti-viral gene expression in PBMCs from HCV patients. PBMCs from both healthy controls and HCV patients showed diverse induction rates of PKR, OAS and MxA following *in vitro* IFN- α stimulation. When ISG expression in PBMCs was examined in HCV patients according to viral genotype, *in vitro* IFN- α stimulation resulted in more diverse patterns of ISG induction in genotype 1 infected patients, than those infected with other genotypes. This was interesting as genotype 1 infected patients exhibit diverse rates of response to clinical IFN- α therapy, whereas the majority of those infected with other genotypes respond positively to IFN- α treatment. Indeed, it has been shown in our lab that HCV does not degrade STAT3 in every HCV patient and 16% of patients still have STAT3 protein expression in their PBMCs despite chronic infection (Genotype 1 infected patients, Dr. Nigel Stevenson, personal communication). Therefore, this could explain why there is a diverse range of ISG induction in PBMCs from HCV infected patients and STAT3 protein expression is

only targeted in some individuals. Ongoing work within our lab aims to examine SNPs in STAT3 that have previously been associated with response to IFN- α therapy in renal cell carcinoma and chronic myeloid leukaemia [254,255], in order to determine what makes an individual susceptible to HCV-mediated STAT3 degradation.

In the liver, HCV infection results in upregulation of endogenous ISGs compared to healthy controls [149]. However, we found no significant differences in endogenous ISG expression in PBMCs from healthy controls and HCV patients. We and others have shown that HCV replicates within PBMCs, albeit at lower levels than within liver (Fig. 4.1)[204]. This could mean that HCV replication is high enough within PBMCs to activate endogenous ISG expression or that HCV is possibly subverting the upregulation of type I IFNs and/or inhibiting IFN- α signalling, to block endogenous ISG expression within PBMCs.

Having observed that HCV patients have diverse rates of response to *in vitro* stimulation of PBMCs with IFN- α , particularly in genotype 1 infected patients, we postulated that patients with lower ISG *in vitro* might also respond poorly to clinical IFN- α therapy. Initially, retrospective analysis of known responders and non-responders was carried out. These were patients who had already undergone IFN- α therapy, therefore the responders were PCR -ve and non-responders PCR +ve at time of *in vitro* analysis. Non-responders had significantly less ISG induction in their PBMCs *in vitro* compared to responders. This was particularly pronounced in genotype 1 infected patients. Endogenous ISG expression levels were similar in responders and non-responders, suggesting that direct IFN- α signalling is compromised. Indeed, further analysis revealed decreased STAT1 and STAT3 activation in PBMCs from non-responders.

Interestingly, non-responders had significantly higher levels of CXCL10 and Trim 21 in their PBMCs. CXCL10 protein levels were higher in the serum of pre-treatment patients who fail to respond to IFN- α therapy [207]. Trim 21 is an E3 ligase that degrades IRF3 and IRF7, thus controlling the induction of type I IFNs [210,211]. Endogenous levels of the type I IFNs, IFN- α and IFN- β , were similar in PBMCs from both responders and non-responders, perhaps suggesting that type I IFN upregulation is inhibited by HCV in PBMCs, possibly through known mechanisms such as inhibition

of RIG-I and TLR3 signalling [135,256]. This could explain why endogenous levels of ISGs are similar in PBMCs from healthy individuals and HCV infected patients, whereas in the liver, HCV infected patients have higher endogenous ISG expression [214].

Previously, our lab demonstrated that proteasomal inhibition could recover STAT3 in PBMCs of HCV patients; therefore we proposed that this recovery of STAT proteins would allow the recovery of ISG expression. PBMCs from non-responders that were treated with a proteasomal inhibitor prior to stimulation with IFN- α , had significantly enhanced ISG expression. To date, only one proteasome inhibitor is used clinically, bortezomib, which is used to treat multiple myeloma and mantle cell lymphoma. Only patients refractory to other treatments and with advanced disease are treated, as the side effects of this drug make it difficult to tolerate [257]. Recently, it has been proposed that rather than blocking the proteasome, specifically targeting E3 ligases would yield better, more tolerable results [174]. This further justifies the use of small molecule inhibitors that specifically target the elongin C and cul-2 binding sites in HCV as a possible future therapeutic strategy to restore responsiveness to IFN- α .

This thesis specifically focused on HCV targeting of STAT3 and not STAT1 for two reasons. First, it had previously been found that HCV can target STAT1 protein expression [166]. Secondly, STAT1 involvement in IFN- α signalling and anti-viral immunity is well established [115], whereas the finding that STAT3 was targeted by HCV to evade innate immune anti-viral responses was completely novel. To further investigate the role of STAT3 in innate immunity, we used a hepatocyte cell line, Huh-7s, and PBMCs from healthy controls or HCV patients. Knock-down of STAT3 expression, with either siRNA or shRNA, in Huh-7 hepatocytes compromised induction of the anti-viral genes PKR and MxA. PBMCs treated with STAT3 siRNA yielded similar results, with both PKR and MxA induction reduced in the absence of STAT3. To confirm that recovery of STAT3 protein expression in PBMCs from non-responder patients was responsible for recovery of ISG expression, STAT3 siRNA was used to knock down STAT3 expression, before proteasomal activity was blocked using MG132. In PBMCs from non-responder patients that were treated with control siRNA, MG132 could restore ISG induction. However, in PBMCs from non-responders treated with STAT3 siRNA, MG132 failed to recover ISG induction, directly linking STAT3 protein

recovery with restoration of ISG induction. This reveals an important, novel role for STAT3 in IFN- α signalling and the upregulation of anti-viral genes.

Our retrospective analysis of patients who failed to demonstrate an SVR to anti-viral therapy found these patients had low anti-viral gene induction *in vitro* in their PBMCs. Therefore, a prospective study was set up to determine whether overall response to clinical IFN- α therapy could be predicted pre-treatment by analysing responsiveness of PBMCs to *in vitro* IFN- α stimulation. Response to combination therapy with IFN- α and ribavirin is suboptimal, with response rates of less than 50% in some instances. The introduction of new protease inhibitors in combination with the current IFN- α and ribavirin regime will increase response rates, especially in patients with the difficult-to-treat genotype 1 [241]. However, new triple therapy has a high cost and additional severe side effects, therefore we also aimed to identify patients pre-treatment, using *in vitro* stimulation of their PBMCs, who would respond to standard IFN- α and ribavirin alone and which patients would have potentially benefitted from addition of protease inhibitors.

Patients infected with genotype 1 of HCV who achieved SVR following IFN- α therapy could be identified pre-treatment through analysis of ISG induction in their PBMCs following *in vitro* IFN- α stimulation. Genotype 1 infected patients who failed to achieve SVR had significantly less expression of ISGs pre-treatment in their PBMCs following IFN- α stimulation. Using gene expression cut-off values, we could positively predict which genotype 1 infected patients who would achieve SVR. This allowed us to identify the patients who would not require triple therapy with new protease inhibitors, which if applied clinically, would yield substantial economic savings and improve quality of life for those patients during therapy. On-treatment viral kinetics at week 4 and 12 (RVR and EVR, respectively) has been shown to be predictive of overall SVR [247]. However, we found no correlation between pre-treatment ISG expression and RVR or EVR. Ongoing work within our lab includes genotyping these pre-treatment patients for IL28B SNPs highly associated with treatment response [5,258]. ISG expression in the liver, with the addition of IL28B SNP genotype, is highly predictive of response to IFN- α therapy [230], therefore investigations will continue into a possible link between ISG expression in PBMCs and IL28B SNPs. Other ongoing work includes efforts to identify a protein biomarker from PBMCs that is a read-out for IFN- α

responsiveness, as this would be a more practically diagnostic test than qRT-PCR. Another investigation is also underway to link pre-treatment STAT3 expression in PBMCs and response to IFN- α therapy. Overall, the aim of is to develop an assay of pre-treatment predictive biomarkers. The future of HCV treatment will be personalised therapeutic regimes, where many predictive marker such as fibrosis score, viral genotype and host innate immune gene polymorphisms, will determine the type and duration of anti-HCV treatment. Our novel finding of *in vitro* IFN- α responsiveness being strongly predictive of clinical response to IFN- α will enhance the development of individualised pre-treatment assessment.

In conclusion, the E2NS2 protein from HCV has immunomodulatory properties capable of inhibiting IFN- α signalling by targeting STAT3, thus blocking induction of anti-viral genes, resulting in chronic HCV infection (Fig. 6.2). Small peptides to block the sites in HCV that mediate inhibition of IFN- α signalling can restore responsiveness to IFN- α and represent a new therapeutic intervention strategy. This also uncovered a previously unknown role for STAT3 in the induction of anti-viral immune responses. Stimulation of PBMCs with IFN- α *in vitro*, both from healthy controls and HCV infected patients, revealed massive diversity in induction of anti-viral genes. In genotype 1 infected patients, those with the lowest induction of anti-viral genes *in vitro* were least likely to respond to clinical IFN- α therapy and would require additional treatment with new protease inhibitors. This predictive assay has significant clinical potential to discriminate between responders and non-responders, thus determining personalised therapeutic options for HCV infected patients.

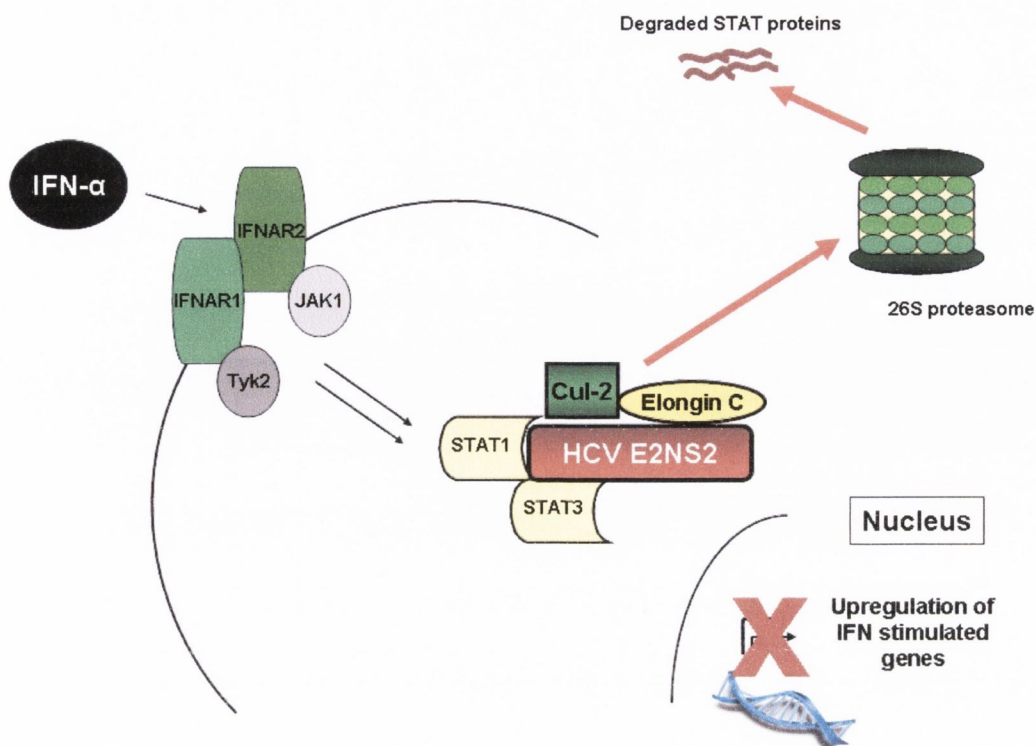


Figure 6.2. HCV degrades STAT proteins via the proteasome to block induction of anti-viral genes

The E2NS2 protein of HCV acts as an E3 ligase to recruit cul-2 and elongin C and allow the virus to target STAT3, and possibly STAT1, for proteasomal degradation. This blocks the upregulation of ISGs critical for effective viral clearance.

6.1 Future work

Although this work has provided a deeper understanding into the interaction of HCV with the anti-viral innate immune system, there are still many aspects that are not understood. In chapter 3. HCV was shown to contain binding sites to allow it to act as an E3 ligase and degrade STAT proteins, therefore inhibiting IFN-α signalling. Blocking the putative elongin C and cul-2 binding sites with short inhibitory peptides could restore IFN-α responsiveness. In a parallel project, Dr. Nigel Stevenson demonstrated the poly-ubiquitination of STAT3 by HCV, both in cell culture and in PBMCs from HCV patients and that HCV associated with elongin C. Although this provides further evidence that HCV is indeed acting as an E3 ligase, the next set of experiments would need to demonstrate a direct interaction between HCV and STAT3.

Should HCV not co-immunoprecipitate with STAT3, then further analysis would be required to investigate whether HCV was targeting STAT3 via another adaptor protein. Another interesting finding of this work was that the elongin C and cul-2 binding sites were highly conserved in genotype 1 of HCV and not genotype 2, 3, 4 and 6. Therefore, as we hypothesised that HCV genotype 1 acts as a strong inhibitor of IFN- α signalling via its E3 ligase potential, analysis and comparison of total STAT protein levels in PBMCs from HCV patients infected with different viral genotypes would experimentally verify whether different genotypes of HCV do indeed have different E3 ligase activity.

Future treatment regime for those infected with HCV will vary in terms of drugs used and duration of treatment and will be based on individual clinical characteristics; therefore a personalised pre-treatment assay will be required to help establish a clinical profile of an individual patient that would determine optimal treatment for that patient. Such pre-treatment assays will be based on many factors such as IL28B genotype, fibrosis score, viral genotype and the age of the patient. As we had found that HCV had a strong inhibitory effect on IFN- α signalling in PBMCs from infected patients, we sought to establish a pre-treatment assay based on responsiveness of PBMCs to *in vitro* IFN- α stimulation and found that in genotype 1 patients, ISG induction in PBMCs was highly predictive of response to therapeutic IFN- α . However, patient numbers recruited for this study were low and although provide an important indication that monitoring an individual's anti-viral immune response can be indicative of response to IFN- α therapy, larger patient numbers need to be recruited to provide more definitive conclusions. Finally, a prospective study of pre-treatment patients would need to be established based on the gene cut-off values we determined to evaluate the overall predictive power of this test.

References

1. Lavanchy D: **Evolving epidemiology of hepatitis C virus.** *Clinical Microbiology and Infection* 2011, **17**:107-115.
2. Liver EAftSot: **EASL Clinical Practice Guidelines: management of hepatitis C virus infection.** *J Hepatol* 2011, **55**:245-264.
3. Der SD, Zhou A, Williams BR, Silverman RH: **Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays.** *Proc Natl Acad Sci U S A* 1998, **95**:15623-15628.
4. Sarwar S, Ryan EJ, Iqbal M, McCormick PA, O'Farrelly C, Hegarty J: **Rapid, early and sustained virological responses in a cohort of Irish patients treated with pegylated interferon and ribavirin for chronic hepatitis C virus infection.** *Ir J Med Sci* 2011.
5. Dring MM, Morrison MH, McSharry BP, Guinan KJ, Hagan R, O'Farrelly C, Gardiner CM, Consortium IHR: **Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection.** *Proc Natl Acad Sci U S A* 2011, **108**:5736-5741.
6. Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, Heathcote J, Edwards AM, McGilvray ID: **Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection.** *Gastroenterology* 2005, **128**:1437-1444.
7. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M: **Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome.** *Science* 1989, **244**:359-362.
8. Simmonds P: **Genetic diversity and evolution of hepatitis C virus—15 years on.** *J Gen Virol* 2004, **85**:3173-3188.
9. Van Etten JL, Lane LC, Dunigan DD: **DNA viruses: the really big ones (giruses).** *Annu Rev Microbiol* 2010, **64**:83-99.
10. Ralston R, Jacobson I, Scull M: **The Conundrum of Relapse in STAT-C Therapy: Does HCV Play the Red Queen or Rip Van Winkle?** *Semin Liver Dis* 2011, **31**:410-419.
11. Moradpour D, Penin F, Rice CM: **Replication of hepatitis C virus.** *Nat Rev Micro* 2007, **5**:453-463.
12. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K: **The lipid droplet is an important organelle for hepatitis C virus production.** *Nat Cell Biol* 2007, **9**:1089-1097.
13. Jean Dubuisson: **Hepatitis C virus proteins.** *World J Gastroenterol.* 2007, **13**:2406-2415.
14. Chou AH, Tsai HF, Wu YY, Hu CY, Hwang LH, Hsu PI, Hsu PN: **Hepatitis C virus core protein modulates TRAIL-mediated apoptosis by enhancing Bid cleavage and activation of mitochondria apoptosis signaling pathway.** *J Immunol* 2005, **174**:2160-2166.
15. Machida K, Tsukiyama-Kohara K, Seike E, Toné S, Shibasaki F, Shimizu M, Takahashi H, Hayashi Y, Funata N, Taya C, et al.: **Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins.** *J Biol Chem* 2001, **276**:12140-12146.
16. Dolganiuc A, Kodys K, Kopasz A, Marshall C, Do T, Romics L, Mandrekar P, Zapp M, Szabo G: **Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation.** *J Immunol* 2003, **170**:5615-5624.
17. Dolganiuc A, Oak S, Kodys K, Golenbock DT, Finberg RW, Kurt-Jones E, Szabo G: **Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation.** *Gastroenterology* 2004, **127**:1513-1524.

18. Dolganiuc A, Norkina O, Kodys K, Catalano D, Bakis G, Marshall C, Mandrekar P, Szabo G: **Viral and host factors induce macrophage activation and loss of toll-like receptor tolerance in chronic HCV infection.** *Gastroenterology* 2007, **133**:1627-1636.
19. Jhaveri R, Kundu P, Shapiro AM, Venkatesan A, Dasgupta A: **Effect of hepatitis C virus core protein on cellular gene expression: specific inhibition of cyclooxygenase 2.** *J Infect Dis* 2005, **191**:1498-1506.
20. Goffard A, Callens N, Bartosch B, Wychowski C, Cosset F-L, Montpellier C, Dubuisson J: **Role of N-Linked Glycans in the Functions of Hepatitis C Virus Envelope Glycoproteins.** *J. Virol.* 2005, **79**:8400-8409.
21. Ndongo N, Berthillon P, Pradat P, Vieux C, Bordes I, Berby F, Maynard M, Zoulim F, Trépo C, Petit MA: **Association of anti-E1E2 antibodies with spontaneous recovery or sustained viral response to therapy in patients infected with hepatitis C virus.** *Hepatology* 2010, **52**:1531-1542.
22. Pestka JM, Zeisel MB, Bläser E, Schürmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, et al.: **Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C.** *Proc Natl Acad Sci U S A* 2007, **104**:6025-6030.
23. Verstrepen BE, Depla E, Rollier CS, Mares G, Drexhage JA, Priem S, Verschoor EJ, Koopman G, Granier C, Dreux M, et al.: **Clearance of genotype 1b hepatitis C virus in chimpanzees in the presence of vaccine-induced E1-neutralizing antibodies.** *J Infect Dis* 2011, **204**:837-844.
24. Varaklioti A, Vassilaki N, Georgopoulou U, Mavromara P: **Alternate translation occurs within the core coding region of the hepatitis C viral genome.** *J Biol Chem* 2002, **277**:17713-17721.
25. Kong J, Deng X, Wang Z, Yang J, Zhang Y, Yu J: **Hepatitis C virus F protein: A double-edged sword in the potential contribution of chronic inflammation to carcinogenesis.** *Mol Med Report* 2009, **2**:461-469.
26. Bain C, Parroche P, Lavergne JP, Duverger B, Vieux C, Dubois V, Komurian-Pradel F, Trépo C, Gebuhrer L, Paranhos-Baccala G, et al.: **Memory T-cell-mediated immune responses specific to an alternative core protein in hepatitis C virus infection.** *J Virol* 2004, **78**:10460-10469.
27. Luik P, Chew C, Aittoniemi J, Chang J, Wentworth P, Dwek RA, Biggin PC, Vénien-Bryan C, Zitzmann N: **The 3-dimensional structure of a hepatitis C virus p7 ion channel by electron microscopy.** *Proc Natl Acad Sci U S A* 2009, **106**:12712-12716.
28. Wozniak AL, Griffin S, Rowlands D, Harris M, Yi M, Lemon SM, Weinman SA: **Intracellular proton conductance of the hepatitis C virus p7 protein and its contribution to infectious virus production.** *PLoS Pathog* 2010, **6**:e1001087.
29. Sakai A, Claire MS, Faulk K, Govindarajan S, Emerson SU, Purcell RH, Bukh J: **The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences.** *Proc Natl Acad Sci U S A* 2003, **100**:11646-11651.
30. Kolykhalov AA, Mihalik K, Feinstone SM, Rice CM: **Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo.** *J Virol* 2000, **74**:2046-2051.
31. Welbourne Sea: **The Hepatitis C Virus NS2/3 Protease.** *Curr. Issues Mol. Biol* 2007, **9**:63-70.
32. Yang X-J, Liu J, Ye L, Liao Q-J, Wu J-G, Gao J-R, She Y-L, Wu Z-H, Ye L-B: **HCV NS2 protein inhibits cell proliferation and induces cell cycle arrest in the S-phase in mammalian cells through down-regulation of cyclin A expression.** *Virus Research* 2006, **121**:134-143.
33. She Yea: **Hepatitis C virus (HCV) NS2 protein up-regulates HCV IRES-dependent translation and down-regulates NS5B RdRp activity.** *Archives of Virology* 2008.

34. Tanji Y, Hijikata M, Satoh S, Kaneko T, Shimotohno K: **Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing.** *J Virol* 1995, **69**:1575-1581.
35. Failla C, Tomei L, De Francesco R: **Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins.** *J Virol* 1994, **68**:3753-3760.
36. Einav S, Elazar M, Danieli T, Glenn JS: **A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication.** *J Virol* 2004, **78**:11288-11295.
37. Macdonald A, Harris M: **Hepatitis C virus NS5A: tales of a promiscuous protein.** *J Gen Virol* 2004, **85**:2485-2502.
38. Okanoue T, Itoh Y, Hashimoto H, Yasui K, Minami M, Takehara T, Tanaka E, Onji M, Toyota J, Chayama K, et al.: **Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study.** *J Gastroenterol* 2009, **44**:952-963.
39. Pascu M, Martus P, Höhne M, Wiedenmann B, Hopf U, Schreier E, Berg T: **Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences.** *Gut* 2004, **53**:1345-1351.
40. Ishii K, Tanaka Y, Yap CC, Aizaki H, Matsuura Y, Miyamura T: **Expression of hepatitis C virus NS5B protein: characterization of its RNA polymerase activity and RNA binding.** *Hepatology* 1999, **29**:1227-1235.
41. Itsui Y, Sakamoto N, Kakinuma S, Nakagawa M, Sekine-Osajima Y, Tasaka-Fujita M, Nishimura-Sakurai Y, Suda G, Karakama Y, Mishima K, et al.: **Antiviral effects of the interferon-induced protein guanylate binding protein 1 and its interaction with the hepatitis C virus NS5B protein.** *Hepatology* 2009, **50**:1727-1737.
42. Choi SH, Park KJ, Ahn BY, Jung G, Lai MM, Hwang SB: **Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor alpha signaling through effects on cellular IkappaB kinase.** *Mol Cell Biol* 2006, **26**:3048-3059.
43. Moriyama M, Kato N, Otsuka M, Shao RX, Taniguchi H, Kawabe T, Omata M: **Interferon-beta is activated by hepatitis C virus NS5B and inhibited by NS4A, NS4B, and NS5A.** *Hepatol Int* 2007, **1**:302-310.
44. Darke PL, Jacobs AR, Waxman L, Kuo LC: **Inhibition of hepatitis C virus NS2/3 processing by NS4A peptides. Implications for control of viral processing.** *J Biol Chem* 1999, **274**:34511-34514.
45. von Hahn T, Rice CM: **Hepatitis C virus entry.** *J Biol Chem* 2008, **283**:3689-3693.
46. Hemler ME: **Tetraspanin functions and associated microdomains.** *Nat Rev Mol Cell Biol* 2005, **6**:801-811.
47. Petracca R, Falugi F, Galli G, Norais N, Rosa D, Campagnoli S, Burgio V, Di Stasio E, Giardina B, Houghton M, et al.: **Structure-function analysis of hepatitis C virus envelope-CD81 binding.** *J Virol* 2000, **74**:4824-4830.
48. Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, Cortese R, Nicosia A, Cosset FL: **Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor.** *J Biol Chem* 2003, **278**:41624-41630.
49. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatzioannou T, McKeating JA, Bieniasz PD, Rice CM: **Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry.** *Nature* 2007, **446**:801-805.
50. Hewitt KJ, Agarwal R, Morin PJ: **The claudin gene family: expression in normal and neoplastic tissues.** *BMC Cancer* 2006, **6**:186.
51. Harris HJ, Farquhar MJ, Mee CJ, Davis C, Reynolds GM, Jennings A, Hu K, Yuan F, Deng H, Hubscher SG, et al.: **CD81 and claudin 1 coreceptor association: role in hepatitis C virus entry.** *J Virol* 2008, **82**:5007-5020.
52. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM: **Human occludin is a hepatitis C virus entry factor required for infection of mouse cells.** *Nature* 2009, **457**:882-886.

53. Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, et al.: **Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate.** *J Biol Chem* 2003, **278**:41003-41012.
54. Barth H, Schnober EK, Zhang F, Linhardt RJ, Depla E, Boson B, Cosset FL, Patel AH, Blum HE, Baumert TF: **Viral and cellular determinants of the hepatitis C virus envelope-heparan sulfate interaction.** *J Virol* 2006, **80**:10579-10590.
55. Rudenko G, Deisenhofer J: **The low-density lipoprotein receptor: ligands, debates and lore.** *Current Opinion in Structural Biology* 2003, **13**:683-689.
56. Germi R, Crance JM, Garin D, Guimet J, Lortat-Jacob H, Ruigrok RW, Zarski JP, Drouet E: **Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption.** *J Med Virol* 2002, **68**:206-215.
57. Hennig BJW, Hellier S, Frodsham AJ, Zhang L, Klenerman P, Knapp S, Wright M, Thomas HC, Thursz M, Hill AVS: **Association of low-density lipoprotein receptor polymorphisms and outcome of hepatitis C infection.** *Genes Immun* 2002, **3**:359-367.
58. ST S, MMC L: **5' and 3' UTR: When translation meets replication.** In *Hepatitis C Viruses: Genomes and Molecular Biology*. Edited by SL T: Horizon Bioscience; 2006.
59. Sabahi A: **Hepatitis C Virus entry: the early steps in the viral replication cycle.** *Virol J* 2009, **6**:117.
60. Bartenschlager R, Cosset F-L, Lohmann V: **Hepatitis C virus replication cycle.** *Journal of Hepatology* 2010, **53**:583-585.
61. Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, Bienz K, Moradpour D: **Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons.** *J Virol* 2003, **77**:5487-5492.
62. Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS: **Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region.** *J Gen Virol* 1993, **74** (Pt 11):2391-2399.
63. Nolte FS, Green AM, Fiebelkorn KR, Caliendo AM, Sturchio C, Grunwald A, Healy M: **Clinical evaluation of two methods for genotyping hepatitis C virus based on analysis of the 5' noncoding region.** *J Clin Microbiol* 2003, **41**:1558-1564.
64. Bartenschlager R, Lohmann V: **Replication of the hepatitis C virus.** *Baillieres Best Pract Res Clin Gastroenterol* 2000, **14**:241-254.
65. Gómez J, Martell M, Quer J, Cabot B, Esteban JI: **Hepatitis C viral quasiespecies.** *J Viral Hepat* 1999, **6**:3-16.
66. Nicot F, Alric L, Barange K, Métivier S, Dramard JM, Combis JM, Castan B, Meurisse JJ, Payen JL, Garipuy D, et al.: **Influence of HCV genotype 1 subtypes on the virus response to PEG interferon alpha-2a plus ribavirin therapy.** *J Med Virol* 2011, **83**:437-444.
67. Chevaliez S, Bouvier-Alias M, Brillet R, Pawlotsky JM: **Hepatitis C virus (HCV) genotype 1 subtype identification in new HCV drug development and future clinical practice.** *PLoS One* 2009, **4**:e8209.
68. Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH, Marcellin P, Muir AJ, Ferenci P, Flisiak R, et al.: **Telaprevir for previously untreated chronic hepatitis C virus infection.** *N Engl J Med* 2011, **364**:2405-2416.
69. Thornton L, Murphy N, Jones L, Connell J, Dooley S, Gavin S, Hunter K, Brennan A: **Determination of the burden of hepatitis C virus infection in Ireland.** *Epidemiol Infect* 2011:1-8.
70. Pol S, Lamorthe B, Thi NT, Thiers V, Carnot F, Zylberberg H, Berthelot P, Bréchet C, Nalpas B: **Retrospective analysis of the impact of HIV infection and alcohol use on chronic hepatitis C in a large cohort of drug users.** *J Hepatol* 1998, **28**:945-950.

71. Kenny-Walsh E: **Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group.** *N Engl J Med* 1999, **340**:1228-1233.
72. Ireland. Health Service Executive., Health Protection Surveillance Centre (Ireland): *National hepatitis C database for infection acquired through blood and blood products : baseline report.* Dublin: Health Protection Surveillance Centre; 2007.
73. Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD: **Analysis of successful immune responses in persons infected with hepatitis C virus.** *J Exp Med* 2000, **191**:1499-1512.
74. Rehmann B, Chang KM, McHutchison JG, Kokka R, Houghton M, Chisari FV: **Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection.** *J Clin Invest* 1996, **98**:1432-1440.
75. Grakoui A, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghayeb J, Murthy KK, Rice CM, Walker CM: **HCV persistence and immune evasion in the absence of memory T cell help.** *Science* 2003, **302**:659-662.
76. Rosen HR, Miner C, Sasaki AW, Lewinsohn DM, Conrad AJ, Bakke A, Bouwer HG, Hinrichs DJ: **Frequencies of HCV-specific effector CD4+ T cells by flow cytometry: correlation with clinical disease stages.** *Hepatology* 2002, **35**:190-198.
77. Petrovic D, Stamataki Z, Dempsey E, Golden-Mason L, Freeley M, Doherty D, Prichard D, Keogh C, Conroy J, Mitchell S, et al.: **Hepatitis C virus targets the T cell secretory machinery as a mechanism of immune evasion.** *Hepatology* 2011, **53**:1846-1853.
78. Takahashi K, Asabe S, Wieland S, Garaigorta U, Gastaminza P, Isogawa M, Chisari FV: **Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection.** *Proc Natl Acad Sci USA* 2010, **107**:7431-7436.
79. Golden-Mason L, Castelblanco N, O'Farrelly C, Rosen HR: **Phenotypic and functional changes of cytotoxic CD56pos natural T cells determine outcome of acute hepatitis C virus infection.** *J Virol* 2007, **81**:9292-9298.
80. Golden-Mason L, Madrigal-Estebas L, McGrath E, Conroy MJ, Ryan EJ, Hegarty JE, O'Farrelly C, Doherty DG: **Altered natural killer cell subset distributions in resolved and persistent hepatitis C virus infection following single source exposure.** *Gut* 2008, **57**:1121-1128.
81. Deignan T, Curry MP, Doherty DG, Golden-Mason L, Volkov Y, Norris S, Nolan N, Traynor O, McEntee G, Hegarty JE, et al.: **Decrease in hepatic CD56(+) T cells and V alpha 24(+) natural killer T cells in chronic hepatitis C viral infection.** *J Hepatol* 2002, **37**:101-108.
82. Kawarabayashi N, Seki S, Hatsuse K, Ohkawa T, Koike Y, Aihara T, Habu Y, Nakagawa R, Ami K, Hiraide H, et al.: **Decrease of CD56+T Cells and Natural Killer Cells in Cirrhotic Livers With Hepatitis C May Be Involved in Their Susceptibility to Hepatocellular Carcinoma.** *Hepatology* 2000, **32**:962-969.
83. Creagh EM, O'Neill LAJ: **TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity.** *Trends in Immunology* 2006, **27**:352-357.
84. O'Neill LA, Bowie AG: **The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.** *Nat Rev Immunol* 2007, **7**:353-364.
85. Takeda K, Akira S: **TLR signaling pathways.** *Semin Immunol* 2004, **16**:3-9.
86. Wilkins C, Gale Jr M: **Recognition of viruses by cytoplasmic sensors.** *Current Opinion in Immunology* 2010, **22**:41-47.
87. Barbalat R, Lau L, Locksley RM, Barton GM: **Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands.** *Nat Immunol* 2009, **10**:1200-1207.
88. Hoffmann M, Zeisel MB, Jilg N, Paranhos-Baccalà G, Stoll-Keller F, Wakita T, Hafkemeyer P, Blum HE, Barth H, Henneke P, et al.: **Toll-like receptor 2 senses hepatitis C virus core protein but not infectious viral particles.** *J Innate Immun* 2009, **1**:446-454.
89. Takeuchi O, Akira S: **Innate immunity to virus infection.** *Immunol Rev* 2009, **227**:75-86.

90. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ: **The nature of the principal type 1 interferon-producing cells in human blood.** *Science* 1999, **284**:1835-1837.
91. Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, et al.: **5'-Triphosphate RNA is the ligand for RIG-I.** *Science* 2006, **314**:994-997.
92. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, et al.: **DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response.** *Nature* 2007, **448**:501-505.
93. Lippmann J, Rothenburg S, Deigendesch N, Eitel J, Meixenberger K, van Laak V, Slevogt H, N'guessan PD, Hippenstiel S, Chakraborty T, et al.: **IFNbeta responses induced by intracellular bacteria or cytosolic DNA in different human cells do not require ZBP1 (DLM-1/DAI).** *Cell Microbiol* 2008, **10**:2579-2588.
94. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA: **AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC.** *Nature* 2009, **458**:514-518.
95. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, et al.: **IFI16 is an innate immune sensor for intracellular DNA.** *Nat Immunol* 2010, **11**:997-1004.
96. ISAACS A, LINDENMANN J: **Virus interference. I. The interferon.** *Proc R Soc Lond B Biol Sci* 1957, **147**:258-267.
97. Pestka S, Krause CD, Walter MR: **Interferons, interferon-like cytokines, and their receptors.** *Immunol Rev* 2004, **202**:8-32.
98. Pestka S: **The human interferon alpha species and receptors.** *Biopolymers* 2000, **55**:254-287.
99. de Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, Silverman RH, Williams BR: **Functional classification of interferon-stimulated genes identified using microarrays.** *J Leukoc Biol* 2001, **69**:912-920.
100. Theofilopoulos AN, Baccala R, Beutler B, Kono DH: **Type I interferons (alpha/beta) in immunity and autoimmunity.** *Annu Rev Immunol* 2005, **23**:307-336.
101. Ogasawara K, Hida S, Weng Y, Saiura A, Sato K, Takayanagi H, Sakaguchi S, Yokochi T, Kodama T, Naitoh M, et al.: **Requirement of the IFN-alpha/beta-induced CXCR3 chemokine signalling for CD8+ T cell activation.** *Genes Cells* 2002, **7**:309-320.
102. Dondi E, Rogge L, Lutfalla G, Uzé G, Pellegrini S: **Down-modulation of responses to type I IFN upon T cell activation.** *J Immunol* 2003, **170**:749-756.
103. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, Cerutti A: **DCs induce CD40-independent immunoglobulin class switching through BlyS and APRIL.** *Nat Immunol* 2002, **3**:822-829.
104. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF: **Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo.** *Immunity* 2001, **14**:461-470.
105. Lutfalla G, Holland SJ, Cinato E, Monneron D, Reboul J, Rogers NC, Smith JM, Stark GR, Gardiner K, Mogensen KE: **Mutant U5A cells are complemented by an interferon-alpha beta receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster.** *EMBO J* 1995, **14**:5100-5108.
106. de Weerd NA, Samarajiwa SA, Hertzog PJ: **Type I interferon receptors: biochemistry and biological functions.** *J Biol Chem* 2007, **282**:20053-20057.
107. Ren F, Hino K, Yamaguchi Y, Okazaki M, Kitase A, Satoh Y, Korenaga M, Okuda M, Okita K: **Hepatitis C virus infection upregulates expression of the type I interferon receptor in human peripheral blood mononuclear cells.** *Hepatol Res* 2003, **26**:15-22.
108. Morita K, Tanaka K, Saito S, Kitamura T, Kiba T, Fujii T, Numata K, Sekihara H: **Expression of interferon receptor genes in the liver as a predictor of interferon response in patients with chronic hepatitis C.** *J Med Virol* 1999, **58**:359-365.

109. Langhans B, Kupfer B, Braunschweiler I, Arndt S, Schulte W, Nischalke HD, Nattermann J, Oldenburg J, Sauerbruch T, Spengler U: **Interferon-lambda serum levels in hepatitis C.** *J Hepatol* 2010.
110. Karaghiosoff M, Neubauer H, Lassnig C, Kovarik P, Schindler H, Pircher H, McCoy B, Bogdan C, Decker T, Brem G, et al.: **Partial Impairment of Cytokine Responses in Tyk2-Deficient Mice.** *Immunity* 2000, **13**:549-560.
111. Rodig SJ, Meraz MA, White JM, Lampe PA, Riley JK, Arthur CD, King KL, Sheehan KCF, Yin L, Pennica D, et al.: **Disruption of the Jak1 Gene Demonstrates Obligatory and Nonredundant Roles of the Jaks in Cytokine-Induced Biologic Responses.** *Cell* 1998, **93**:373-383.
112. Decker T, Kovarik P: **Serine phosphorylation of STATs.** *Oncogene* 2000, **19**:2628-2637.
113. Mitchell TJ, John S: **Signal transducer and activator of transcription (STAT) signalling and T-cell lymphomas.** *Immunology* 2005, **114**:301-312.
114. Durbin JE, Hackenmiller R, Simon MC, Levy DE: **Targeted Disruption of the Mouse Stat1 Gene Results in Compromised Innate Immunity to Viral Disease.** *Cell* 1996, **84**:443-450.
115. Meraz MA, White JM, Sheehan KCF, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, et al.: **Targeted Disruption of the Stat1 Gene in Mice Reveals Unexpected Physiologic Specificity in the JAK-STAT Signaling Pathway.** *Cell* 1996, **84**:431-442.
116. Park C, Li S, Cha E, Schindler C: **Immune response in Stat2 knockout mice.** *Immunity* 2000, **13**:795-804.
117. Yang CH, Murti A, Pfeffer LM: **STAT3 complements defects in an interferon-resistant cell line: evidence for an essential role for STAT3 in interferon signaling and biological activities.** *Proc Natl Acad Sci U S A* 1998, **95**:5568-5572.
118. Ho HH, Ivashkiv LB: **Role of STAT3 in type I interferon responses. Negative regulation of STAT1-dependent inflammatory gene activation.** *J Biol Chem* 2006, **281**:14111-14118.
119. Akira S: **Functional roles of STAT family proteins: lessons from knockout mice.** *Stem Cells* 1999, **17**:138-146.
120. Schindler C, Plumlee C: **Interferons pen the JAK-STAT pathway.** *Seminars in Cell & Developmental Biology* 2008, **19**:311-318.
121. Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA: **Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection.** *Science* 2002, **297**:2063-2066.
122. Uddin S, Lekmine F, Sassano A, Rui H, Fish EN, Platanias LC: **Role of Stat5 in Type I interferon-signaling and transcriptional regulation.** *Biochemical and Biophysical Research Communications* 2003, **308**:325-330.
123. Lemaire PA, Anderson E, Lary J, Cole JL: **Mechanism of PKR Activation by dsRNA.** *J Mol Biol* 2008, **381**:351-360.
124. Toroney R, Nallagatla SR, Boyer JA, Cameron CE, Bevilacqua PC: **Regulation of PKR by HCV IRES RNA: importance of domain II and NS5A.** *J Mol Biol* 2010, **400**:393-412.
125. Malathi K, Dong B, Gale M, Silverman RH: **Small self-RNA generated by RNase L amplifies antiviral innate immunity.** *Nature* 2007, **448**:816-819.
126. Haller O, Staeheli P, Kochs G: **Interferon-induced Mx proteins in antiviral host defense.** *Biochimie* 2007, **89**:812-818.
127. Sadler AJ, Williams BRG: **Interferon-inducible antiviral effectors.** *Nat Rev Immunol* 2008, **8**:559-568.
128. Stevenson NJ, Murphy AG, Bourke NM, Keogh CA, Hegarty JE, O'Farrelly C: **Ribavirin Enhances IFN- α Signalling and MxA Expression: A Novel Immune Modulation Mechanism during Treatment of HCV.** *PLoS One* 2011, **6**:e27866.
129. Hansen TH, Bouvier M: **MHC class I antigen presentation: learning from viral evasion strategies.** *Nat Rev Immunol* 2009, **9**:503-513.

130. Favoreel HW, Van de Walle GR, Nauwynck HJ, Pensaert MB: **Virus complement evasion strategies.** *J Gen Virol* 2003, **84**:1-15.
131. Stack J, Haga IR, Schröder M, Bartlett NW, Maloney G, Reading PC, Fitzgerald KA, Smith GL, Bowie AG: **Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence.** *J Exp Med* 2005, **201**:1007-1018.
132. Harte MT, Haga IR, Maloney G, Gray P, Reading PC, Bartlett NW, Smith GL, Bowie A, O'Neill LA: **The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense.** *J Exp Med* 2003, **197**:343-351.
133. Wu J, Meng Z, Jiang M, Pei R, Trippler M, Broering R, Bucci A, Sowa J-P, Dittmer U, Yang D, et al.: **Hepatitis B virus suppresses toll-like receptor-mediated innate immune responses in murine parenchymal and nonparenchymal liver cells.** *Hepatology* 2009, **49**:1132-1140.
134. Wilson JR, de Sessions PF, Leon MA, Scholle F: **West Nile virus nonstructural protein 1 inhibits TLR3 signal transduction.** *J Virol* 2008, **82**:8262-8271.
135. Li K, Foy E, Ferreón JC, Nakamura M, Ferreón AC, Ikeda M, Ray SC, Gale M, Lemon SM: **Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF.** *Proc Natl Acad Sci U S A* 2005, **102**:2992-2997.
136. Abe T, Kaname Y, Hamamoto I, Tsuda Y, Wen X, Tagawa S, Moriishi K, Takeuchi O, Kawai T, Kanto T, et al.: **Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines.** *J Virol* 2007, **81**:8953-8966.
137. Barral PM, Morrison JM, Drahos J, Gupta P, Sarkar D, Fisher PB, Racaniello VR: **MDA-5 is cleaved in poliovirus-infected cells.** *J Virol* 2007, **81**:3677-3684.
138. Gack MU, Albrecht RA, Urano T, Inn K-S, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, García-Sastre A: **Influenza A Virus NS1 Targets the Ubiquitin Ligase TRIM25 to Evade Recognition by the Host Viral RNA Sensor RIG-I.** *Cell Host & Microbe* 2009, **5**:439-449.
139. Unterholzner L, Sumner RP, Baran M, Ren H, Mansur DS, Bourke NM, Randow F, Smith GL, Bowie AG: **Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7.** *PLoS Pathog* 2011, **7**:e1002247.
140. Varga ZT, Ramos I, Hai R, Schmolke M, García-Sastre A, Fernandez-Sesma A, Palese P: **The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein.** *PLoS Pathog* 2011, **7**:e1002067.
141. Qu L, Feng Z, Yamane D, Liang Y, Lanford RE, Li K, Lemon SM: **Disruption of TLR3 signaling due to cleavage of TRIF by the hepatitis A virus protease-polymerase processing intermediate, 3CD.** *PLoS Pathog* 2011, **7**:e1002169.
142. Lu LL, Puri M, Horvath CM, Sen GC: **Select paramyxoviral V proteins inhibit IRF3 activation by acting as alternative substrates for inhibitor of kappaB kinase epsilon (IKKe)/TBK1.** *J Biol Chem* 2008, **283**:14269-14276.
143. Melroe GT, Silva L, Schaffer PA, Knipe DM: **Recruitment of activated IRF-3 and CBP/p300 to herpes simplex virus ICP0 nuclear foci: Potential role in blocking IFN-beta induction.** *Virology* 2007, **360**:305-321.
144. Symons JA, Alcami A, Smith GL: **Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity.** *Cell* 1995, **81**:551-560.
145. Li S, Labrecque S, Gauzzi MC, Cuddihy AR, Wong AH, Pellegrini S, Matlashewski GJ, Koromilas AE: **The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha.** *Oncogene* 1999, **18**:5727-5737.
146. Parisien JP, Lau JF, Rodriguez JJ, Sullivan BM, Moscona A, Parks GD, Lamb RA, Horvath CM: **The V protein of human parainfluenza virus 2 antagonizes type I**

- interferon responses by destabilizing signal transducer and activator of transcription 2. *Virology* 2001, **283**:230-239.
147. Elliott J, Lynch OT, Suessmuth Y, Qian P, Boyd CR, Burrows JF, Buick R, Stevenson NJ, Touzelet O, Gadina M, et al.: **Respiratory Syncytial Virus NS1 Protein Degrades STAT2 by Using the Elongin-Cullin E3 Ligase.** *J. Virol.* 2007, **81**:3428-3436.
 148. Ulane CM, Rodriguez JJ, Parisien J-P, Horvath CM: **STAT3 Ubiquitylation and Degradation by Mumps Virus Suppress Cytokine and Oncogene Signaling.** *J. Virol.* 2003, **77**:6385-6393.
 149. Helbig KJ, Yip E, McCartney EM, Eyre NS, Beard MR: **A screening method for identifying disruptions in interferon signaling reveals HCV NS3/4a disrupts Stat-1 phosphorylation.** *Antiviral Research* 2008, **77**:169-176.
 150. Lin W, Kim SS, Yeung E, Kamegaya Y, Blackard JT, Kim KA, Holtzman MJ, Chung RT: **Hepatitis C Virus Core Protein Blocks Interferon Signaling by Interaction with the STAT1 SH2 Domain.** *J. Virol.* 2006, **80**:9226-9235.
 151. Duong FH, Filipowicz M, Tripodi M, La Monica N, Heim MH: **Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A.** *Gastroenterology* 2004, **126**:263-277.
 152. Adolfo G-S: **Mechanisms of inhibition of the host interferon α/β -mediated antiviral responses by viruses.** *Microbes and Infection* 2002, **4**:647-655.
 153. Gale Jr MJ, Korth MJ, Tang NM, Tan S-L, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG: **Evidence That Hepatitis C Virus Resistance to Interferon Is Mediated through Repression of the PKR Protein Kinase by the Nonstructural 5A Protein.** *Virology* 1997, **230**:217-227.
 154. Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM: **Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein.** *Science* 1999, **285**:107-110.
 155. Sánchez R, Mohr I: **Inhibition of cellular 2'-5' oligoadenylate synthetase by the herpes simplex virus type 1 Us11 protein.** *J Virol* 2007, **81**:3455-3464.
 156. Taguchi T, Nagano-Fujii M, Akutsu M, Kadoya H, Ohgimoto S, Ishido S, Hotta H: **Hepatitis C virus NS5A protein interacts with 2',5'-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner.** *J Gen Virol* 2004, **85**:959-969.
 157. Hoenen A, Liu W, Kochs G, Khromykh AA, Mackenzie JM: **West Nile virus-induced cytoplasmic membrane structures provide partial protection against the interferon-induced antiviral MxA protein.** *J Gen Virol* 2007, **88**:3013-3017.
 158. Veronese FM, Pasut G: **PEGylation, successful approach to drug delivery.** *Drug Discovery Today* 2005, **10**:1451-1458.
 159. Dixit NM, Layden-Almer JE, Layden TJ, Perelson AS: **Modelling how ribavirin improves interferon response rates in hepatitis C virus infection.** *Nature* 2004, **432**:922-924.
 160. Thomas E, Feld JJ, Li Q, Hu Z, Fried MW, Liang TJ: **Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models.** *Hepatology* 2010.
 161. Colin C, Lanoir D, Touzet S, Meyaud-Kraemer L, Bailly F, Trepo C, Group H: **Sensitivity and specificity of third-generation hepatitis C virus antibody detection assays: an analysis of the literature.** *J Viral Hepat* 2001, **8**:87-95.
 162. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J: **Growth of human hepatoma cells lines with differentiated functions in chemically defined medium.** *Cancer Res* 1982, **42**:3858-3863.
 163. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al.: **Clustal W and Clustal X version 2.0.** *Bioinformatics* 2007, **23**:2947-2948.
 164. Plataniias LC: **Mechanisms of type-I- and type-II-interferon-mediated signalling.** *Nat Rev Immunol* 2005, **5**:375-386.

165. Lemon SM: **Induction and evasion of innate antiviral responses by hepatitis C virus.** *J Biol Chem* 2010, **285**:22741-22747.
166. Lin W, Choe WH, Hiasa Y, Kamegaya Y, Blackard JT, Schmidt EV, Chung RT: **Hepatitis C virus expression suppresses interferon signaling by degrading STAT1.** *Gastroenterology* 2005, **128**:1034-1041.
167. Lan K-H, Lan K-L, Lee W-P, Sheu M-L, Chen M-Y, Lee Y-L, Yen S-H, Chang F-Y, Lee S-D: **HCV NS5A inhibits interferon- α signaling through suppression of STAT1 phosphorylation in hepatocyte-derived cell lines.** *Journal of Hepatology* 2007, **46**:759-767.
168. Christen V, Treves S, Duong FH, Heim MH: **Activation of endoplasmic reticulum stress response by hepatitis viruses up-regulates protein phosphatase 2A.** *Hepatology* 2007, **46**:558-565.
169. Pickart CM: **Mechanisms underlying ubiquitination.** *Annu Rev Biochem* 2001, **70**:503-533.
170. Jin J, Li X, Gygi SP, Harper JW: **Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging.** *Nature* 2007, **447**:1135-1138.
171. Li W, Bengtson MH, Ulbrich A, Matsuda A, Reddy VA, Orth A, Chanda SK, Batalov S, Joazeiro CA: **Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling.** *PLoS One* 2008, **3**:e1487.
172. Miller J, Gordon C: **The regulation of proteasome degradation by multi-ubiquitin chain binding proteins.** *FEBS Letters* 2005, **579**:3224-3230.
173. Deshaies RJ, Joazeiro CA: **RING domain E3 ubiquitin ligases.** *Annu Rev Biochem* 2009, **78**:399-434.
174. Kling J: **New twists on proteasome inhibitors.** *Nat Biotech* 2010, **28**:1236-1238.
175. Kamura T, Maenaka K, Kotoshiba S, Matsumoto M, Kohda D, Conaway RC, Conaway JW, Nakayama KI: **VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases.** *Genes Dev* 2004, **18**:3055-3065.
176. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG: **HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing.** *Science* 2001, **292**:464-468.
177. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ: **The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.** *Nature* 1999, **399**:271-275.
178. Clifford SC, Astuti D, Hooper L, Maxwell PH, Ratcliffe PJ, Maher ER: **The pVHL-associated SCF ubiquitin ligase complex: molecular genetic analysis of elongin B and C, Rbx1 and HIF-1 α in renal cell carcinoma.** *Oncogene* 2001, **20**:5067-5074.
179. Shuai K, Liu B: **Regulation of JAK-STAT signalling in the immune system.** *Nat Rev Immunol* 2003, **3**:900-911.
180. Yu X, Yu Y, Liu B, Luo K, Kong W, Mao P, Yu XF: **Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex.** *Science* 2003, **302**:1056-1060.
181. Luo K, Ehrlich E, Xiao Z, Zhang W, Ketner G, Yu XF: **Adenovirus E4orf6 assembles with Cullin5-ElonginB-ElonginC E3 ubiquitin ligase through an HIV/SIV Vif-like BC-box to regulate p53.** *FASEB J* 2007, **21**:1742-1750.
182. Horvath CM: **Weapons of STAT destruction. Interferon evasion by paramyxovirus V protein.** *Eur J Biochem* 2004, **271**:4621-4628.
183. Kuiken C, Yusim K, Boykin L, Richardson R: **The Los Alamos hepatitis C sequence database.** *Bioinformatics* 2005, **21**:379-384.
184. Zhu H, Shang X, Terada N, Liu C: **STAT3 induces anti-hepatitis C viral activity in liver cells.** *Biochem Biophys Res Commun* 2004, **324**:518-528.

185. Ohh M, Takagi Y, Aso T, Stebbins CE, Pavletich NP, Zbar B, Conaway RC, Conaway JW, Kaelin WG: **Synthetic peptides define critical contacts between elongin C, elongin B, and the von Hippel-Lindau protein.** *J Clin Invest* 1999, **104**:1583-1591.
186. Owsianka A, Clayton RF, Loomis-Price LD, McKeating JA, Patel AH: **Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2.** *J Gen Virol* 2001, **82**:1877-1883.
187. Lin C, Lindenbach BD, Prágai BM, McCourt DW, Rice CM: **Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini.** *J Virol* 1994, **68**:5063-5073.
188. Isherwood BJ, Patel AH: **Analysis of the processing and transmembrane topology of the E2p7 protein of hepatitis C virus.** *J Gen Virol* 2005, **86**:667-676.
189. Bartenschlager R, Lohmann V: **Replication of hepatitis C virus.** *J Gen Virol* 2000, **81**:1631-1648.
190. Komurian-Pradel F, Perret M, Deiman B, Sodoyer M, Lotteau V, Paranhos-Baccalà G, André P: **Strand specific quantitative real-time PCR to study replication of hepatitis C virus genome.** *J Virol Methods* 2004, **116**:103-106.
191. Blackard JT, Kemmer N, Sherman KE: **Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences.** *Hepatology* 2006, **44**:15-22.
192. Laporte J, Bain C, Maurel P, Inchauspe G, Agut H, Cahour A: **Differential distribution and internal translation efficiency of hepatitis C virus quasiespecies present in dendritic and liver cells.** *Blood* 2003, **101**:52-57.
193. Lee WM, Polson JE, Carney DS, Sahin B, Gale M: **Reemergence of hepatitis C virus after 8.5 years in a patient with hypogammaglobulinemia: evidence for an occult viral reservoir.** *J Infect Dis* 2005, **192**:1088-1092.
194. Lin A, Thadareddy A, Goldstein MJ, Lake-Bakaar G: **Immune suppression leading to hepatitis C virus re-emergence after sustained virological response.** *J Med Virol* 2008, **80**:1720-1722.
195. Baré P: **Hepatitis C virus and peripheral blood mononuclear cell reservoirs** Patricia Baré. *World J Hepatol* 2009, **1**:67-71.
196. Li Y, Wang X, Douglas SD, Metzger DS, Woody G, Zhang T, Song L, Ho WZ: **CD8+ T cell depletion amplifies hepatitis C virus replication in peripheral blood mononuclear cells.** *J Infect Dis* 2005, **192**:1093-1101.
197. Baré P, Massud I, Parodi C, Belmonte L, García G, Nebel MC, Corti M, Pinto MT, Bianco RP, Bracco MM, et al.: **Continuous release of hepatitis C virus (HCV) by peripheral blood mononuclear cells and B-lymphoblastoid cell-line cultures derived from HCV-infected patients.** *J Gen Virol* 2005, **86**:1717-1727.
198. Zimmerer JM, Lesinski GB, Ruppert AS, Radmacher MD, Noble C, Kendra K, Walker MJ, Carson WE: **Gene expression profiling reveals similarities between the in vitro and in vivo responses of immune effector cells to IFN-alpha.** *Clin Cancer Res* 2008, **14**:5900-5906.
199. Pugnale P, Herrmann E, Neumann AU, Pawlotsky JM, Schalm SW, Ferrari C, Homburger Y, Zeuzem S, Negro F, Group D-HS: **Hepatitis C viral kinetics in plasma and peripheral blood mononuclear cells during pegylated interferon-alpha2a/ribavirin therapy.** *J Hepatol* 2008, **48**:932-938.
200. Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI: **Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C.** *J Virol* 2004, **78**:5867-5874.
201. Radkowski M, Gallegos-Orozco JF, Jablonska J, Colby TV, Walewska-Zielecka B, Kubicka J, Wilkinson J, Adair D, Rakela J, Laskus T: **Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C.** *Hepatology* 2005, **41**:106-114.
202. Forns X, García-Retortillo M, Serrano T, Feliu A, Suarez F, de la Mata M, García-Valdecasas JC, Navasa M, Rimola A, Rodés J: **Antiviral therapy of patients with**

- decompensated cirrhosis to prevent recurrence of hepatitis C after liver transplantation. *J Hepatol* 2003, **39**:389-396.
203. Lanford RE, Guerra B, Bigger CB, Lee H, Chavez D, Brasky KM: **Lack of response to exogenous interferon-alpha in the liver of chimpanzees chronically infected with hepatitis C virus.** *Hepatology* 2007, **46**:999-1008.
 204. Lerat H, Rumin S, Habersetzer F, Berby F, Traubad MA, Trépo C, Inchauspé G: **In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype.** *Blood* 1998, **91**:3841-3849.
 205. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, Heim MH: **Interferon signaling and treatment outcome in chronic hepatitis C.** *Proc Natl Acad Sci U S A* 2008, **105**:7034-7039.
 206. Lee EY, Lee Z-H, Song YW: **CXCL10 and autoimmune diseases.** *Autoimmunity Reviews* 2009, **8**:379-383.
 207. Lagging M, Romero AI, Westin J, Norkrans G, Dhillon AP, Pawlotsky JM, Zeuzem S, von Wagner M, Negro F, Schalm SW, et al.: **IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection.** *Hepatology* 2006, **44**:1617-1625.
 208. Schulte-Pelkum J, Fritzler M, Mahler M: **Latest update on the Ro/SS-A autoantibody system.** *Autoimmunity Reviews* 2009, **8**:632-637.
 209. Ceribelli A, Cavazzana I, Cattaneo R, Franceschini F: **Hepatitis C virus infection and primary Sjögren's Syndrome: A clinical and serologic description of 9 patients.** *Autoimmunity Reviews* 2008, **8**:92-94.
 210. Higgs R, Ní Gabhann J, Ben Larbi N, Breen EP, Fitzgerald KA, Jefferies CA: **The E3 ubiquitin ligase Ro52 negatively regulates IFN-beta production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3.** *J Immunol* 2008, **181**:1780-1786.
 211. Higgs R, Lazzari E, Wynne C, Ní Gabhann J, Espinosa A, Wahren-Herlenius M, Jefferies CA: **Self protection from anti-viral responses--Ro52 promotes degradation of the transcription factor IRF7 downstream of the viral Toll-Like receptors.** *PLoS One* 2010, **5**:e11776.
 212. Mallery DL, McEwan WA, Bidgood SR, Towers GJ, Johnson CM, James LC: **Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21).** *Proc Natl Acad Sci U S A* 2010, **107**:19985-19990.
 213. Nordstokke D, Zumbo B, Cairns S, Saklofske D: **The operating characteristics of the nonparametric Levene test for equal variances with assessment and evaluation data.** In *Practical Assessment, Research and Evaluation*. Edited by. Online; 2011. vol 16.]
 214. Helbig KJ, Lau DT, Semendric L, Harley HA, Beard MR: **Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector.** *Hepatology* 2005, **42**:702-710.
 215. Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, Lemon SM, Lanford RE: **Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees.** *J Virol* 2004, **78**:13779-13792.
 216. Shen Q, Zhang R, Bhat NR: **MAP kinase regulation of IP10/CXCL10 chemokine gene expression in microglial cells.** *Brain Res* 2006, **1086**:9-16.
 217. Casrouge A, Decalf J, Ahloulay M, Lababidi C, Mansour H, Vallet-Pichard A, Mallet V, Mottez E, Mapes J, Fontanet A, et al.: **Evidence for an antagonist form of the chemokine CXCL10 in patients chronically infected with HCV.** *J Clin Invest* 2011, **121**:308-317.
 218. Mihm S, Frese M, Meier V, Wietzke-Braun P, Scharf JG, Bartenschlager R, Ramadori G: **Interferon type I gene expression in chronic hepatitis C.** *Lab Invest* 2004, **84**:1148-1159.

219. Meier V, Mihm S, Ramadori G: **Interferon-alpha therapy does not modulate hepatic expression of classical type I interferon inducible genes.** *J Med Virol* 2008, **80**:1912-1918.
220. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçalves FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, et al.: **Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection.** *N Engl J Med* 2002, **347**:975-982.
221. Pawlotsky JM: **Hepatitis C virus resistance to antiviral therapy.** *Hepatology* 2000, **32**:889-896.
222. Hayashi K, Katano Y, Ishigami M, Itoh A, Hirooka Y, Nakano I, Urano F, Yoshioka K, Toyoda H, Kumada T, et al.: **Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy.** *J Viral Hepat* 2011, **18**:280-286.
223. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK: **Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial.** *Lancet* 2001, **358**:958-965.
224. Villa E, Karampatou A, Cammà C, Di Leo A, Luongo M, Ferrari A, Petta S, Losi L, Taliani G, Trande P, et al.: **Early Menopause Is Associated With Lack of Response to Antiviral Therapy in Women With Chronic Hepatitis C.** *Gastroenterology* 2010.
225. Bräu N, Bini EJ, Currie S, Shen H, Schmidt WN, King PD, Ho SB, Cheung RC, Hu KQ, Anand BS, et al.: **Black patients with chronic hepatitis C have a lower sustained viral response rate than non-Blacks with genotype 1, but the same with genotypes 2/3, and this is not explained by more frequent dose reductions of interferon and ribavirin*.** *J Viral Hepat* 2006, **13**:242-249.
226. Ghany MG, Strader DB, Thomas DL, Seeff LB, Diseases AAftSoL: **Diagnosis, management, and treatment of hepatitis C: an update.** *Hepatology* 2009, **49**:1335-1374.
227. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'hUigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, et al.: **Genetic variation in IL28B and spontaneous clearance of hepatitis C virus.** *Nature* 2009, **461**:798-801.
228. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, Bochud M, Battegay M, Bernasconi E, Borovicka J, et al.: **Genetic Variation in IL28B Is Associated With Chronic Hepatitis C and Treatment Failure: A Genome-Wide Association Study.** *Gastroenterology* 2010, **138**:1338-1345.e1337.
229. Darling JM, Aerssens J, Fanning G, McHutchison JG, Goldstein DB, Thompson AJ, Shianna KV, Afdhal NH, Hudson ML, Howell CD, et al.: **Quantitation of pretreatment serum interferon-γ-inducible protein-10 improves the predictive value of an IL28B gene polymorphism for hepatitis C treatment response.** *Hepatology* 2011, **53**:14-22.
230. Dill MT, Duong FH, Vogt JE, Bibert S, Bochud PY, Terracciano L, Papassotiropoulos A, Roth V, Heim MH: **Interferon-Induced Gene Expression Is a Stronger Predictor of Treatment Response Than IL28B Genotype in Patients With Hepatitis C.** *Gastroenterology* 2010.
231. Seeff LB, Everson GT, Morgan TR, Curto TM, Lee WM, Ghany MG, Shiffman ML, Fontana RJ, Di Bisceglie AM, Bonkovsky HL, et al.: **Complication rate of percutaneous liver biopsies among persons with advanced chronic liver disease in the HALT-C trial.** *Clin Gastroenterol Hepatol* 2010, **8**:877-883.
232. Hauser P, Khosla J, Aurora H, Laurin J, Kling MA, Hill J, Gulati M, Thornton AJ, Schultz RL, Valentine AD, et al.: **A prospective study of the incidence and open-label treatment of interferon-induced major depressive disorder in patients with hepatitis C.** *Mol Psychiatry* 2002, **7**:942-947.
233. Fried MW: **Side effects of therapy of hepatitis C and their management.** *Hepatology* 2002, **36**:S237-244.

234. Bacon BR, Gordon SC, Lawitz E, Marcellin P, Vierling JM, Zeuzem S, Poordad F, Goodman ZD, Sings HL, Boparai N, et al.: **Boceprevir for previously treated chronic HCV genotype 1 infection.** *N Engl J Med* 2011, **364**:1207-1217.
235. Poordad F, McCone J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, Jacobson IM, Reddy KR, Goodman ZD, Boparai N, et al.: **Boceprevir for untreated chronic HCV genotype 1 infection.** *N Engl J Med* 2011, **364**:1195-1206.
236. Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, Roberts S, Focaccia R, Younossi Z, Foster GR, Horban A, et al.: **Telaprevir for retreatment of HCV infection.** *N Engl J Med* 2011, **364**:2417-2428.
237. Bruno R, Cima S, Maiocchi L, Sacchi P: **Forthcoming challenges in the management of direct-acting antiviral agents (DAAs) for hepatitis C.** *Dig Liver Dis* 2011, **43**:337-344.
238. Halfon P, Locarnini S: **Hepatitis C virus resistance to protease inhibitors.** *J Hepatol* 2011, **55**:192-206.
239. Dahl G, Sandström A, Akerblom E, Danielson UH: **Resistance profiling of hepatitis C virus protease inhibitors using full-length NS3.** *Antivir Ther* 2007, **12**:733-740.
240. Gane E: **Future hepatitis C virus treatment: interferon-sparing combinations.** *Liver Int* 2011, **31 Suppl 1**:62-67.
241. Asselah T, Marcellin P: **New direct-acting antivirals' combination for the treatment of chronic hepatitis C.** *Liver Int* 2011, **31 Suppl 1**:68-77.
242. Cheruvattath R, Rosati MJ, Gautam M, Vargas HE, Rakela J, Balan V: **Pegylated interferon and ribavirin failures: is retreatment an option?** *Dig Dis Sci* 2007, **52**:732-736.
243. Ceriotti F, Henny J, Queralto J, Ziyu S, Özarda Y, Chen B, Boyd JC, Panteghini M, (C-RIDL) ICoRIaDL, (C-RSE) CoRSfE: **Common reference intervals for aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ -glutamyl transferase (GGT) in serum: results from an IFCC multicenter study.** *Clin Chem Lab Med* 2010, **48**:1593-1601.
244. Ji X, Cheung R, Cooper S, Li Q, Greenberg HB, He XS: **Interferon alfa regulated gene expression in patients initiating interferon treatment for chronic hepatitis C.** *Hepatology* 2003, **37**:610-621.
245. He XS, Ji X, Hale MB, Cheung R, Ahmed A, Guo Y, Nolan GP, Pfeffer LM, Wright TL, Risch N, et al.: **Global transcriptional response to interferon is a determinant of HCV treatment outcome and is modified by race.** *Hepatology* 2006, **44**:352-359.
246. Medrano J, Barreiro P, Resino S, Tuma P, Rodríguez V, Vispo E, Labarga P, Madejón A, García-Samaniego J, Jiménez-Nácher I, et al.: **Rate and timing of hepatitis C virus relapse after a successful course of pegylated interferon plus ribavirin in HIV-infected and HIV-uninfected patients.** *Clin Infect Dis* 2009, **49**:1397-1401.
247. Yu JW, Wang GQ, Sun LJ, Li XG, Li SC: **Predictive value of rapid virological response and early virological response on sustained virological response in HCV patients treated with pegylated interferon alpha-2a and ribavirin.** *J Gastroenterol Hepatol* 2007, **22**:832-836.
248. Zweig MH, Campbell G: **Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine.** *Clin Chem* 1993, **39**:561-577.
249. Pham TN, Coffin CS, Michalak TI: **Occult hepatitis C virus infection: what does it mean?** *Liver Int* 2010, **30**:502-511.
250. Butera D, Marukian S, Iwamaye AE, Hembrador E, Chambers TJ, Di Bisceglie AM, Charles ED, Talal AH, Jacobson IM, Rice CM, et al.: **Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C.** *Blood* 2005, **106**:1175-1182.
251. Tohme RA, Holmberg SD: **Is sexual contact a major mode of hepatitis C virus transmission?** *Hepatology* 2010, **52**:1497-1505.
252. Lambers FA, Prins M, Thomas X, Molenkamp R, Kwa D, Brinkman K, van der Meer JT, Schinkel J, group MMOSoAIwhCs: **Alarming incidence of hepatitis C virus re-**

infection after treatment of sexually acquired acute hepatitis C virus infection in HIV-infected MSM. *AIDS* 2011, 25:F21-27.

253. Takaoka A, Yanai H: **Interferon signalling network in innate defence. *Cell Microbiol* 2006, 8:907-922.**
254. Ito N, Eto M, Nakamura E, Takahashi A, Tsukamoto T, Toma H, Nakazawa H, Hirao Y, Uemura H, Kagawa S, et al.: **STAT3 polymorphism predicts interferon-alfa response in patients with metastatic renal cell carcinoma. *J Clin Oncol* 2007, 25:2785-2791.**
255. Kreil S, Waghorn K, Ernst T, Chase A, White H, Hehlmann R, Reiter A, Hochhaus A, Cross NC, Group GCS: **A polymorphism associated with STAT3 expression and response of chronic myeloid leukemia to interferon alpha. *Haematologica* 2010, 95:148-152.**
256. Lin R, Lacoste J, Nakhaei P, Sun Q, Yang L, Paz S, Wilkinson P, Julkunen I, Vitour D, Meurs E, et al.: **Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage. *J Virol* 2006, 80:6072-6083.**
257. Roussou M, Kastritis E, Migkou M, Psimenou E, Grapsa I, Matsouka C, Barmparousi D, Terpos E, Dimopoulos MA: **Treatment of patients with multiple myeloma complicated by renal failure with bortezomib-based regimens. *Leuk Lymphoma* 2008, 49:890-895.**
258. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, et al.: **Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009, 461:399-401.**