

Innate and Adaptive Immune Responses in Chronic HCV infection

Lynn B. Dustin

University of Oxford

Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences

Kennedy Institute of Rheumatology

Peter Medawar Building for Pathogen Research

South Parks Road

Oxford OX1 3SY, United Kingdom

Correspondence: Lynn.Dustin@Kennedy.ox.ac.uk

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1. Abstract

Hepatitis C virus (HCV) remains a public health problem of global importance, even in the era of potent directly-acting antiviral drugs. In this chapter, I discuss immune response to acute and chronic HCV infection. The outcome of HCV infection is influenced by viral strategies that limit or delay the initiation of innate antiviral responses. This delay may enable HCV to establish widespread infection long before the host mounts effective T and B cell responses. HCV's genetic agility, resulting from its high rate of replication and its error prone replication mechanism, enables it to evade immune recognition. Adaptive immune responses fail to keep up with changing viral epitopes. Neutralizing antibody epitopes may be hidden by decoy structures, glycans, and lipoproteins. T cell responses fail due to changing epitope sequences and due to exhaustion, a phenomenon that may have evolved to limit immune-mediated pathology. Despite these difficulties, innate and adaptive immune mechanisms do impact HCV replication. Immune-mediated clearance of infection is possible, occurring in 20-50% of people who contract the disease. New developments raise hopes for effective immunological interventions to prevent or treat HCV infection.

14 **2. Introduction**

15 Although estimates vary, it is believed that between 130 million and 200 million people worldwide are
16 persistently infected with the hepatitis C virus, HCV (1-3). There is not yet an approved prophylactic
17 vaccine. HCV is transmitted through percutaneous contact with infected blood. In most developed countries,
18 blood screening has virtually eliminated the risk of infection through blood and blood products, but HCV
19 transmission remains high in developing countries and also among people who inject drugs. Occupational,
20 nosocomial, and vertical transmission are all observed, and sexual transmission of HCV may occur in some
21 settings. Acute HCV infection may be asymptomatic or the symptoms may be nonspecific; thus, people may
22 not know they are infected until many years later, when significant liver damage has occurred (4). Over 20-
23 30 years, 15-30% of those chronically infected with HCV may develop long-term complications including
24 cirrhosis; some of those can go on to develop hepatocellular carcinoma and/or end-stage liver disease (4, 5).
25 HCV infection is now the leading indication for liver transplantation (6). Patients who harbor HCV at the
26 time of transplantation experience recurrent infection of the grafted liver, frequently leading to accelerated
27 fibrosis and cirrhosis (6).

28
29 Deaths from HCV now outstrip those from HIV infection in the developed world, and HCV infection
30 increases mortality from other causes (7, 8). HCV complicates the outcome and treatment of other infectious
31 diseases, and other infectious diseases complicate HCV pathogenesis and treatment. Thus, an estimated 20-
32 30% of people with HIV infection worldwide are co-infected with HCV. HIV/HCV co-infection is
33 associated with higher HCV viral loads, increased HCV chronicity, reduced response to anti-HCV therapy,
34 and accelerated liver damage compared to HCV-mono-infection. Co-infected patients are also more likely to
35 suffer kidney and neurocognitive disease than are HIV-mono-infected patients, and HCV co-infection can
36 impact antiretroviral therapy for HIV (5, 9, 10). Hepatitis B virus (HBV) can exacerbate liver disease due to
37 persistent HCV infection, while super-infection with HCV can exacerbate liver disease due to chronic HBV
38 infection (11). Co-infection with HCV and liver-tropic parasites such as *Schistoma mansoni* may also lead to
39 more rapid and severe liver disease than either pathogen alone (12). The immunopathogenic mechanisms of
40 co-infection are still poorly understood and require additional study.

41

42 The landscape for HCV treatment is changing rapidly, and new directly-acting antiviral (**DAA**) drugs offer
43 the hope that most patients who are treated can be cured (5, 13-16). At this time, however, most patients
44 have not been either diagnosed or treated (17, 18). Among the numerous barriers to treatment are ignorance
45 of infection status, uneven healthcare access, concern about side effects, and high drug prices (19). In
46 addition, antiviral treatment will not immediately reverse liver disease in the millions of patients who have
47 been infected for decades and in whom the burden of HCV-related liver disease will continue to increase
48 dramatically in the coming years (20).

49

50 **2. The goal of a vaccine**

51 The availability of DAAs will not eliminate HCV as a global health problem. Ultimately, an effective,
52 widely available vaccine will be needed to curb ongoing HCV transmission (21-23). While most HCV-
53 infected patients progress to chronic hepatitis with persistent viremia, a significant minority (20-50%) of
54 patients mount a successful immune response to HCV, resulting in **spontaneous resolution** of infection;
55 recovery rates differ depending on factors such as age, race, sex, and genetics (5, 24-28). Thus, immune
56 mediated control is possible. Can we stimulate a successful immune response, and thus protection from HCV
57 persistence, with a vaccine? Several challenges have hindered vaccine development work to date. HCV
58 presents extensive genetic diversity: there are seven major genotypes, whose nucleotide sequences differ
59 from each other by 30% or more, and dozens of subtypes differing by at least 15% (29). Recent work has
60 demonstrated that T cell immunity to HCV is likely to be genotype or even isolate-specific, even in subjects
61 who spontaneously resolve infection (30). The inter-genotype and inter-subtype genetic diversity is
62 compounded by HCV's rapid evolution within each infected host. Also important, the correlates of
63 protection from HCV infection are still incompletely understood. Discouragingly, those fortunate people
64 who clear HCV infection without treatment have incomplete protection from re-infection (31-33).

65

66 **3. Hepatitis C virus, antiviral therapy, and sites of replication**

67 HCV is an enveloped RNA virus in the family Flaviviridae. Its genomic structure and replication
68 mechanisms are detailed in several excellent recent reviews (16, 34-36). HCV has a single-stranded, positive
69 sense RNA genome of approximately 9.6 kb (**Figure 1**). The RNA lacks 5' cap and 3' poly-A structures;

70 translation occurs via an internal ribosomal entry site (IRES) that hijacks the ribosome to enable cap-
71 independent protein translation. HCV RNA encodes a single polyprotein of approximately 3010 amino acids,
72 which is processed co- and post-translationally by host and viral proteases to release ten mature proteins.
73 These proteins are Core (capsid), the envelope glycoproteins E1 and E2, and seven non-structural proteins:
74 p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The assembled virus is believed to incorporate HCV RNA
75 plus core, E1, and E2 (37); p7 and NS2 are not required for HCV RNA replication but are required for virus
76 assembly and egress; NS3-5B together make up the viral replicase while also contributing to assembly (36).
77 RNA replication proceeds through a negative-sense RNA template. HCV's NS5B RNA-dependent RNA
78 polymerase lacks proofreading activity. As a result, the viral RNA changes rapidly and circulates within each
79 infected host as a quasispecies--a swarm of related viral sequences. This availability of a diverse pool of viral
80 sequences provides the raw material for rapid evolution under selection from host immune pressures and
81 antiviral drugs.

82
83 Until recently, the gold standard for antiviral therapy involved prolonged treatment (24-48 weeks) with
84 injected, PEGylated IFN α preparations plus the nucleoside analog, ribavirin. The treatment was difficult to
85 tolerate and often ineffective. DAAs have dramatically changed the landscape for HCV patients, but the new
86 drugs are expensive. DAAs target key steps in HCV replication and assembly. A detailed discussion of this
87 topic is beyond the scope of this chapter, but the reader is referred to a number of excellent recent reviews (5,
88 15, 16). The first DAAs that came to market targeted the NS3-4A protease, blocking cleavage of the
89 polyprotein and perhaps also of cellular targets. These first-generation DAAs, including Boceprevir and
90 Telaprevir, were most effective when administered in combination with PEGylated IFN α and ribavirin; they
91 were not equally effective in all HCV genotypes; and viral resistance to these drugs could be achieved with a
92 small number of mutations (this is termed a low **barrier to resistance**). Subsequent generations of NS3-4A
93 protease inhibitors are expected to have broader HCV genotype coverage and a higher genetic barrier to
94 resistance. Development of drugs targeting other HCV proteins has raised hopes for the availability of an all-
95 oral, IFN-free treatment regimen. Drugs that target NS5A (for example, Daclatasvir and Ledipasvir) have
96 high potency, but some have a relatively low barrier to resistance. Drugs that target the NS5B polymerase
97 include nucleoside analogs such as Sofosbuvir, which block polymerase activity in all HCV genotypes, and

98 non-nucleoside drugs that may act by allosteric mechanisms. The non-nucleoside drugs target specific HCV
99 genotypes. Combinations of these drugs act synergistically to inhibit HCV replication. All-oral drug
100 combinations targeting 2 or 3 viral targets have shown extremely high potency in clinical trials. Additional
101 drugs, targeting host factors required for HCV replication, are also in development. Host targets of these
102 drugs include the microRNA miR-122, cyclophilin, and molecules required in HCV entry (16).

103

104 The primary site of HCV replication is the hepatocyte. Replication in blood cells and other tissues, if it
105 occurs, is likely to be limited by several factors; these other cell types have not yielded robust and
106 reproducible in vitro systems for HCV study. Importantly, HCV replication is dependent on the liver-specific
107 miRNA, miR-122 (38-40). HCV particle assembly is tightly linked to host lipid and lipoprotein biosynthetic
108 pathways (36, 37), and circulating HCV associates with host lipoproteins. Infectious HCV behaves as a lipo-
109 viral particle (36, 41, 42) that takes advantage of hepatocyte lipoprotein capture mechanisms to gain entry
110 into susceptible cells (42, 43). Many of the host factors involved in HCV entry are not expressed at
111 significant levels in blood cells (44). Virus may be taken into many cell types without productive infection.
112 Examination of biopsy material shows that infected hepatocytes are distributed in clusters of 4-50 cells (45),
113 suggesting that HCV may spread efficiently from an infected “founder” cell to its closest neighbors (45-47).

114

115 **4. Detecting and responding to HCV: Innate antiviral responses**

116 Virus replication generates numerous pathogen-associated molecular patterns (**PAMPs**), and these in turn
117 stimulate IFN and IFN-stimulated gene (**ISG**) expression. ISGs mediate numerous direct antiviral effects.
118 IFNs, ISGs, inflammatory cytokines, and other signals derived from infected cells contribute to the initiation
119 and regulation of adaptive immune responses. HCV, like many viral pathogens, has evolved strategies to
120 blunt the innate antiviral response (48, 49). Thus, HCV RNA replication takes place in specialized, enclosed
121 structures that may hide PAMPs from cellular detection (36). As discussed in this section, the viral NS3-4A
122 protease specifically targets some proteins involved in virus detection. Other viral strategies to block IFN
123 induction and signaling have also been proposed (reviewed in (50-52)). However, as discussed below,
124 despite HCV’s ability to disable virus-sensing mechanisms within infected cells, antiviral pathways are
125 initiated soon after virus infection and persist in the chronically-infected liver.

126

127 **4.1. Recognition of viral RNA**

128 HCV RNA has a phosphorylated 5' end and a poly U/UC sequence near its 3' end. These PAMPs together
129 facilitate HCV RNA recognition by the cytosolic RNA binding protein, retinoic acid induced gene-I (**RIG-I**)
130 (53-56). RNA-bound RIG-I triggers ubiquitination reactions and the formation of signaling complexes that
131 ultimately activate the mitochondrial antiviral signaling protein (**MAVS**) (57). MAVS resides on
132 intracellular membranes of mitochondria, mitochondrial-associated membranes near the endoplasmic
133 reticulum, and peroxisomes. When activated, MAVS forms prion-like aggregates which recruit additional
134 ubiquitin ligase activities (58). These ubiquitin ligases catalyze the production of K63 polyubiquitin chains,
135 which facilitates protein-protein interactions that then activate the downstream interferon response factor-3
136 (**IRF3**) and **NFκB** pathways required for IFN induction (58, 59) (**Figure 2A**). Of interest, the various
137 subcellular locations of MAVS are associated with distinct downstream activities (48, 60). Peroxisome-
138 associated MAVS may be key to HCV recognition in hepatocytes because of its unique ability to stimulate
139 **IFNλ** gene expression (60). HCV's NS3-4A protease cleaves MAVS, releasing it from subcellular
140 membranes, and can thereby prevent MAVS-mediated induction of IFN expression in HCV-infected cells
141 (61, 62). MAVS cleavage has been observed in the HCV-infected liver (63, 64). However, RIG-I can
142 recognize incoming viral RNA as it emerges from the nucleocapsid—before any viral replication or genome
143 translation (65). This early recognition may explain, in part, why HCV-infected cells mount innate responses
144 despite the presence of cleaved MAVS.

145

146 HCV RNA folds into complex structures featuring numerous stem-loops that can also act as PAMPs (**Figure**
147 **1**). These include the internal ribosomal entry site (IRES) near the 5' end of the HCV genome. Innate
148 recognition of this PAMP may paradoxically reduce antiviral responses (66). As early as 2 hours post
149 infection, HCV IRES binding to protein kinase R (PKR) reportedly stimulates a signaling cascade involving
150 MAVS, TRAF3, and IRF3, but not RIG-I, to induce IFN-β and ISG expression (67). PKR inhibits host cell
151 protein translation by phosphorylating and inactivating the eukaryotic translation initiation factor, eIF2α.
152 Such inhibition may reduce the translation of antiviral ISGs, without inhibiting the (eIF2α-independent)

153 IRES-mediated translation of HCV RNA (66, 68). By activating PKR, HCV may also trigger ISG15
154 expression (67). Higher ISG15 levels are associated with poor response to IFN-based HCV therapies (69,
155 70), and ISG15 could promote HCV replication by interfering with the ubiquitination reactions needed for
156 RIG-I signaling (67).

157

158 HCV RNA replication entails production of a double-stranded RNA (dsRNA) intermediate; dsRNA binds
159 and activates the endosomal Toll-like receptor 3 (**TLR3**). TLR3 engagement stimulates IFN gene expression
160 via the adaptor molecule, TIR-containing adaptor inducing IFN β (**TRIF**). TRIF signals via ubiquitin ligase-
161 dependent pathways to activate IRF3 and NF κ B (reviewed in (49, 59, 71)) (**Figure 2B**). HCV's NS3-4A
162 protease may disable TLR3 signaling by cleaving TRIF, potentially disabling this pathway in infected cells
163 (49, 72). Some questions remain about the role of TLR3, and of TRIF cleavage, in innate HCV recognition.
164 TLR3's RNA binding domains are localized in the endosomes rather than the cytosol; thus, they may not
165 have access to HCV PAMPs within the infected cell. TLR3 may recognize HCV RNA taken up from
166 neighboring infected cells or possibly in a cell-autonomous manner through autophagy. It is not clear
167 whether or how NS3-4A-mediated TRIF cleavage affects the detection of HCV RNA by bystander or
168 immune cells, since few if any of these cells would be infected—therefore, these cells are unlikely to express
169 NS3-4A.

170

171 **4.2 IFNs and ISGs**

172 Despite the cleavage of MAVS and TRIF, it is clear that HCV stimulates innate antiviral pathways during
173 acute and chronic infection. In acutely infected chimpanzees, hepatic ISG expression coincides with partial
174 control of HCV replication (73-75). The sources and types of the IFN involved were not determined in these
175 studies (73-76). Importantly, hepatocytes can express both type I (α/β) and type III (λ) IFNs, and can
176 respond to these as well as to type II (γ) IFN.

177

178 Recent studies have shown that hepatocytes preferentially express type III IFNs in response to HCV
179 infection, and that this expression is observed even in the presence of MAVS cleavage (77-81). HCV-

180 infected primary liver cells produce IFN λ protein at levels sufficient to limit HCV replication (77, 81).

181 Single nucleotide polymorphisms (SNPs) in and near the type III IFN genes are associated with the outcome

182 of primary HCV infection (82, 83), IFN α -based antiviral therapy (84-88), and quantitative variation in ISG

183 expression in vivo (89, 90) and in vitro (80). The type III IFN receptor, IFNLR, is preferentially expressed in

184 epithelial tissues including the liver parenchyma (reviewed in (87)). Type III IFNs are encoded in a cluster

185 on the long arm of human chromosome 19 (reviewed in (87, 88)). The genes are *IFNL1*, encoding the protein

186 IFN λ 1 (previously IL-29); *IFNL2*, encoding the protein IFN λ 2 (previously IL-28A), *IFNL3*, encoding IFN λ 3

187 (previously IL-28B), and the more recently discovered *IFNL4*, encoding IFN λ 4.

188

189 How does polymorphism in this region influence HCV infection? Higher intrahepatic ISG expression at

190 baseline is paradoxically associated with reduced likelihood of response to IFN α -based therapeutic regimens

191 (91-93). The non-protective SNPs are associated with relatively higher ISG expression in patients with HCV

192 infection (89), but not in healthy subjects (90). Because the important SNPs were close to the *IFNL3* gene,

193 initial studies focused on their effects on *IFNL3* mRNA expression (85, 86, 89, 92, 94) or stability (95).

194 Results were inconsistent (88). In 2013, researchers identified a novel gene, now termed *IFNL4*, in the

195 *IFNL3* region; this gene is transiently expressed in human hepatocytes following stimulation with a double-

196 stranded RNA PAMP (96). The protective *IFNL3*/IL28B SNPs are in linkage disequilibrium with

197 polymorphisms in the *IFNL4* gene. Specifically, a protective TT allele at rs368234815 disrupts the *IFNL4*

198 open reading frame, abolishing protein expression, while the non-protective *IFNL4* Δ G allele at this site

199 results in an intact open reading frame (96). The *IFNL4* Δ G allele is believed to be the ancestral allele (88).

200 The high frequency of the TT allele in some populations suggests that these populations are descended from

201 survivors of some (presumably infectious) event that selected against expression of IFN λ 4 (88). The non-

202 protective *IFNL4* Δ G allele is associated with development of persistent HCV infection (97). A different

203 *IFNL4* polymorphism, encoding a variant with diminished IFN activity, is also associated with

204 improvements in spontaneous and treatment-induced clearance (98). *IFNL4* is more distantly related to the

205 other members of the *IFNL* gene family, but may have a similar protein structure (99) and has demonstrable

206 antiviral activity (99). Despite the strong evidence that ability to express IFN λ 4 contributes to development

207 of persistent HCV infection, increased baseline ISG expression, and reduced efficacy of IFN α -based antiviral
208 therapy, the mechanisms by which IFN λ 4 may influence these disparate outcomes remain uncertain.

209

210 In vivo, IFNs produced by other cell types also undoubtedly contribute to the hepatic ISG response. Thus,
211 dendritic cells (DCs) patrolling the liver may produce type I (100-102) and type III (103) IFNs following
212 recognition of HCV RNA in exosomes or debris from infected hepatocytes. Recent work suggests that a very
213 small subset of myeloid DCs expressing BDCA3/CD141 may play a key role in production of type III IFNs
214 in response to HCV or debris from infected cells; type III IFN production by these cells is dependent on
215 TLR3 signaling (104-106). Liver endothelial cells may also produce IFNs and amplify local IFN responses
216 following detection of virus or debris (107). Natural killer cells and T cells also play key roles, as discussed
217 in later sections of this chapter. IFN γ derived from activated T cells, in particular, is associated with viral
218 clearance.

219

220 Which ISGs are expressed, and how do they impact HCV replication? There are more than 300 ISGs,
221 mediating diverse antiviral functions as discussed in several excellent recent reviews (49, 76, 108-110).
222 Although the type I and type III IFNs signal through distinct receptors, they induce similar sets of ISGs
223 (albeit with somewhat different kinetics (87, 111, 112)). Among the ISGs expressed following HCV
224 infection, several have potential activity against HCV (49, 77, 80, 113). These include oligoadenylate
225 synthetases (OAS1 and OAS2), which synthesize 2'-5' oligoadenylate that can, in turn, activate RNase L to
226 cleave HCV RNA; ISG20, an exonuclease; IFIT1 (ISG56), which may inhibit HCV IRES-mediated
227 polyprotein translation; IRF1, IRF7, STAT1, RIG-I, and MDA-5, which may amplify IFN expression or
228 response; RSAD2 (viperin) which may act on HCV through its interaction with lipid droplets (114); IFITM1
229 and IFITM3, which may act to inhibit viral entry and fusion. While specific individual ISGs are able to
230 reduce HCV replication *in vitro*, multiple ISGs likely operate synergistically within infected cells.

231

232 **4.3 Why doesn't innate immunity clear HCV infection?**

233 Importantly, the cycle of virus entry, virus detection, ISG induction, and innate antiviral response continues
234 throughout chronic infection. HCV and ISG RNA can be found in the same cells in vitro (80) and during
235 chronic infection in vivo (46) (but see (47)). The observation that ISG expression in the liver correlates with
236 a sharp drop in viremia during acute HCV infection (73-75) strongly suggests that innate mechanisms
237 control HCV infection in vivo. These observations raise the question of why endogenous IFNs and ISGs
238 don't cure HCV infection. Indeed, higher ISG expression before the start of therapy is associated with a poor
239 outcome in IFN-based antiviral therapy (91-93). High intrahepatic ISG expression is now believed to
240 correlate with polymorphisms in the IFN λ locus (89). Stochastic variability in IFN induction and/or
241 responsiveness between cells may provide HCV with a continuous supply of susceptible cells with reduced
242 ISG expression (80, 115, 116). Other mechanisms may also contribute. HCV-activated PKR could shut down
243 translation of host cell ISG mRNAs without affecting HCV translation (68); to learn whether this mechanism
244 operates in the infected liver, it will be necessary to define, at the protein level, which ISGs are expressed in
245 infected cells and neighboring uninfected cells. Researchers have proposed that HCV proteins, notably core,
246 E2, and NS5A, may interfere with IFN signal transduction or block specific ISG functions (reviewed in (48-
247 50)); of note, however, many of these studies have depended on overexpression of single HCV proteins in
248 transfected cells, and have not been confirmed in infected cells or in patients. Finally, reduced HCV
249 replication may be advantageous for a persisting virus because it limits antigen display and therefore T cell
250 recognition, thus preventing development of fulminant liver disease and extending host survival.

251

252 **5. Innate immune cells in chronic HCV infection**

253 The liver environment is rich in cells that bridge innate and adaptive immunity. Here, I will touch briefly on
254 natural killer (NK) cells, dendritic cells (DCs), and cells in the monocyte-macrophage lineage. For a recent
255 review on these and other innate cells in the liver, the reader is referred to (117).

256

257 **5.1 NK cells**

258 NK cells are abundant in the liver in health and during HCV infection (118, 119). NK cell activation is
259 mediated in part by integration of information from a diverse array of activating and inhibitory receptor-
260 ligand pairs (120). Genetic polymorphisms in the numbers and types of these receptors, and their ligands,

mandate that developing NK cells must be educated or “licensed” to function in each individual (120, 121). Of note, some genetic polymorphisms that affect NK cell activation thresholds are reported to correlate with spontaneous and treatment-induced clearance of HCV (122, 123).

NK cells are among the earliest immune responders to viral infection, but the roles of NK cells in control of HCV are still poorly understood (119). They may contribute to viral control through secretion of cytokines, including IFN α , IFN γ , and TNF α , that can drive ISG expression, inhibit viral replication, promote DC maturation, and promote release of chemokines that recruit lymphoid and inflammatory cells (reviewed in (50)). NK cells may also mediate direct lysis of infected hepatocytes, but it is not known whether this activity occurs in patients, how infected cells are recognized, or whether such lysis has a significant impact on viral loads. NK cells may modulate adaptive immune responses by killing antigen-presenting cells and activated CD4⁺ T cells (120); whether this mechanism operates during HCV infection is not yet known. Through early antiviral activities, NK cells may protect T cells from exhaustion due to high antigen levels (reviewed in (121)). NK cells are activated during acute HCV infection, arguing against reports that HCV envelope proteins inhibit NK cell function (119, 124, 125). In vitro studies also refute the hypothesis that HCV somehow inhibits NK cell function (126).

There is a great deal of literature describing possible alterations in NK cell phenotype, subset distribution, and function during HCV infection, but these reports are sometimes contradictory (119, 127, 128). Altered expression of specific activating and inhibitory receptors has been expressed in some studies, but the mechanisms of these alterations are not yet certain. Functional NK cell subsets include a CD56^{bright}/CD16^{negative} subset that produces IFN γ and a CD56^{dim}/CD16^{positive} subset that mediates cytotoxic activity. Altered ratios of cytotoxic to IFN γ -producing subsets have been reported in peripheral blood NK cells in patients with persistent HCV infection. Increased levels of a functionally deficient NK subset (CD56^{negative}/CD16^{positive}) are also reported. It is unclear whether altered NK cell subset distribution represents dysfunction, specific activation in the liver environment, or a protective mechanism that limits NK-mediated liver damage.

288

289 **5.2. Dendritic cells (DCs)**

290 DC subsets (129-131) influence the outcome of HCV infection through production of IFNs and other
291 cytokines that directly affect viral replication and influence T cell activation, and through potent antigen
292 presentation to T cells. Of the many DC subsets, three in particular have been studied for their role in HCV
293 infection: plasmacytoid DCs (pDCs), which produce high levels of IFNs; myeloid or classical DCs (mDCs),
294 which play essential roles in antigen presentation; and a minor population termed mDC2, identified by the
295 expression of BDCA3/CD141, which may be especially potent IFN λ producers (106). DCs may change their
296 phenotypes and functions in response to different signals, thus there may be some fluidity among DC subsets
297 (131).

298

299 Recognition of distinct PAMPs drives DCs to produce different cytokines, and these in turn promote
300 different downstream immune responses (reviewed in (131)). pDCs preferentially recognize HCV via TLR7
301 (which binds single-stranded RNA), and produce higher levels of type I IFNs. The mDC2 subset appears to
302 preferentially recognize HCV via TLR3 sensing of double-stranded RNA, and to produce particularly high
303 levels of type III IFNs. The IFNs can act on hepatocytes and also on myriad other cell populations. DCs
304 activated through engagement of the endosomal nucleic acid receptors TLR3, TLR7, or TLR9 produce IL-
305 12; this cytokine supports TH1 differentiation and subsequent competence to produce IFN γ . IL-18 and IL-27
306 from DCs can synergize with IL-12 to promote TH1 development. Other PAMPs, including those binding
307 TLR2, induce IL-10, which supports TH2 differentiation. There are reports that various HCV structural and
308 non-structural proteins stimulate TLR2 on DCs, resulting in functional changes (reviewed in (117)). Some
309 PAMPs, notably those derived from fungi, can promote release of IL-6 and IL-23, which support TH17
310 differentiation. Some PAMPs induce production of IL-10, retinoic acid, and TGF- β , supporting Treg
311 differentiation. DCs can also release IL-15, which supports T cell survival. Type I IFN, IL-12, and IL-15
312 derived from DCs support NK cell activation, cytotoxicity, and survival (117).

313

314 HCV's effects on DCs are controversial. Peripheral blood DCs are reduced in number in HCV patients
315 (reviewed in (50, 117)), but this quantitative reduction is not accompanied by functional deficit (132-134).

DCs may be reduced in the blood because of accumulation in the liver (135). Patients with HCV infection do not suffer from global immunological impairment, arguing against the notion that HCV exerts a broad inhibitory effect on DCs. Some groups report that DC function is impaired or cytokine production profiles altered in HCV patients (136-138). Cell culture-produced HCV was reported to inhibit IFN α production by DCs stimulated with TLR9 ligands (139); other studied functions were unaffected, and inhibition was independent of HCV infection or replicative capacity. Disruption of DC functions has been described in various vitro systems utilizing overexpression or high doses of individual HCV proteins (reviewed in (117)); the significance of these changes in vivo is not known. Arguing against an important role for HCV non-structural proteins within DCs, I would note that it is not clear that DCs support HCV infection. DCs do not express the full complement of entry factors required for infection of hepatocytes (44). The levels of HCV RNA associated with DCs in chronically infected patients are well below one RNA copy per cell (137, 140), suggesting that at most a minor population of patient DCs support any HCV replication. DC uptake of HCV for antigen presentation occurs independently of HCV entry factors (141). DCs may capture HCV (without productive infection) in part through the C-type lectin DC-SIGN (142, 143), perhaps permitting them to deliver HCV to susceptible cells.

331

An important role for DC activation and function is shown in prospective studies of people exposed to HCV through illicit drug use. Subjects who mounted a successful immune response, resulting in spontaneous resolution of infection, had reduced numbers of mature pDCs and increased evidence of mDC activation, including increased cytokine production in response to a diverse array of TLR ligands, increased expression of the T cell costimulator CD86, and reduced expression of inhibitory ligands such as PD-L1 and PD-L2 (144).

338

339 **5.3 Monocytes, macrophages, and Kupffer cells**

Monocytes, macrophages, and Kupffer cells (macrophages resident in the hepatic sinusoids) are abundant in the liver and are thought to play key roles in hepatic inflammation (145). These cell types express a variety of receptors that can detect pathogens and PAMPs at the cell surface and following internalization (145). Receptors at the cell surface include DC-SIGN, mannose receptor, and scavenger receptors. Human Kupffer

cells are reported to produce inflammatory cytokines, notably IL-1 β and IL-18, following interaction with HCV or HCV-infected cells (146-149). HCV replication is not required for this effect. The roles of IL-1 β and IL-18 in chronic infection are not yet well understood, but they may contribute to intrahepatic and systemic inflammation, and increase production of IFN γ by NK cells (148). Kupffer cells, monocytes, and macrophages in the liver can modulate inflammation, immunity, and fibrosis through release of a host of other cytokines, including IL-10, IL-6, IL-12, TNF α and TGF β , as well as reactive oxygen species (145). These cells may also present antigen to T cells; their expression of costimulatory ligands such as CD86, and co-inhibitory ligands including PD-L1, PD-L2, and galectin 9 may influence subsequent T cell activation and function (145).

353

354 **6. T lymphocytes in HCV infection**

T cells mediate both protective and pathological roles in HCV infection. Analysis of acute resolving infections suggests that T cells may control HCV infection both through IFN γ production and by killing infected cells. Spontaneous resolution of HCV infection follows the appearance in the liver of T cells expressing IFN γ (50, 74, 75, 150-153). In successful immune responses, these cells must target a broad array of HCV epitopes in order to reduce viral immune escape. These broadly-reactive T cell responses are sustained through spontaneous HCV clearance and for years afterward. In contrast, in infections that progress to chronicity, initial broadly directed T cell responses collapse (**Figure 3**). Both CD4⁺ and CD8⁺ T cell subsets are essential for resolution of infection, as demonstrated by studies in which chimpanzees that had previously cleared HCV were re-infected after depletion of one subset or the other (154, 155).

364

IFN γ stimulates an antiviral program in hepatocytes, inhibiting HCV replication without killing infected cells; this mechanism allows limited numbers of antigen-specific T cells to mediate control, and offers the advantage of hepatocyte survival (156). However, the data support a key role for T cell-mediated killing of infected hepatocytes. T cell perforin expression, and hepatocyte apoptosis, correlated with spontaneous clearance of HCV in experimentally infected chimpanzees (157). Unlike IFN γ -dependent mechanisms, cytolytic control of HCV replication is likely to depend on larger numbers of T lymphocytes (156). As discussed below, persistent HCV infection is associated with **exhaustion** of HCV-specific T cells. Cytolytic

372 activity is lost early in the development of T cell exhaustion, while IFN γ production may persist (158). If
373 cytolysis of infected hepatocytes is required for resolution of infection, then loss of T cell proliferative
374 capacity, associated with “stunned” HCV-specific T cells (159), could contribute to the low rate of
375 spontaneous clearance.

376

377 Adaptive immunity exerts control over HCV infection even in persistent infection; thus, immunosuppressive
378 therapy, immunodeficiencies, and HIV co-infection all exacerbate HCV-driven liver disease (4). Immunity
379 to HCV re-infection is possible (31, 160). Unfortunately, however, even vigorous T cell responses after
380 spontaneous clearance of HCV infection do not confer complete protection against subsequent challenge
381 with homologous or heterologous HCV strains (161-163). Indeed, chimpanzees experimentally exposed to
382 trace quantities of HCV showed induction of regulatory T cells and poor subsequent responses to HCV
383 infection (164).

384

385 Immune responses to HCV are not always beneficial. While infected hepatocytes may have reduced lifespan
386 (45, 165), HCV is not thought to be directly cytopathic. T cells, rather than virus, may be responsible for
387 liver damage in acute infection (74, 166). In chronic infection, most of the inflammatory T cells infiltrating
388 the liver are not even specific for HCV (167-169).

389

390 **6.1. What happens to T cells in persistent HCV infection?**

391 Immune responses to HCV are slow to develop even in infections that resolve without treatment (**Figure 3**).
392 This delay may be due in part to HCV’s effects on innate immune signaling, as discussed in the previous
393 section. As HCV infection progresses to persistence, the initial, broadly directed HCV-specific CD4⁺ and
394 CD8⁺ T cell response weakens. The number of epitopes targeted is reduced due to failure of epitope-specific
395 T cell populations and to viral evolution. The waning of HCV-specific T cell function may be an adaptive
396 response that reduces T cell-mediated tissue damage in conditions of persistent, high antigen load; reduced
397 viral loads may permit T cells to recover their function (170).

398

399 **6.2. CD4⁺ T cell failure**

HCV-specific CD4⁺ T cell help is lost as infection persists, and this loss contributes to the decline in function of HCV-specific CD8⁺ T cells. The pathways leading to loss of CD4⁺ T cell help are still not understood (171). HCV-specific CD4⁺ T cells are observable during acute infections, even those that progress to chronicity (166, 172, 173). However, HCV-specific CD4⁺ T cells are scarce in established chronic infection (172, 174). Studies in mice with chronic viral infection have shown that activated NK cells may facilitate viral persistence and CD4⁺ T cell failure by killing responding CD4⁺ T cells (152, 175, 176). Whether such a mechanism contributes to loss of HCV-specific CD4⁺ T cells in humans is not yet known. HCV-specific CD4⁺ T cells have lower expression of the **IL-7 receptor alpha chain, CD127**, during infections that progress to chronicity compared to infections that resolve without treatment (177). Given the importance of IL-7 in memory T cell survival and turnover (178), loss of this signal may contribute to the disappearance of CD4⁺ T cell responses to HCV. Patients with chronically-evolving HCV infection were reported to have increased expression of a negative regulator, Tim-3, on CD4⁺ T cells, but this observation was not limited to HCV-reactive T cells (179). HCV-specific CD4⁺ T cells express the inhibitory co-receptor programmed death-1 (PD-1) (180, 181) during acute and chronic infection. It has been reported that blockade of PD-1, TGFβ and IL-10 (181) or IL-2 supplementation (173) may partially restore functionality of HCV-specific CD4⁺ T cells. Regulatory T cells may play a role in HCV persistence; in a cohort of injection drug users with acute HCV infection, increased regulatory T cell function and decreased Th17 function correlated with progression to chronicity (182). Importantly, it is not clear whether these changes are causes or effects of CD4⁺ T cell failure in persisting HCV infection. Eliminating HCV-specific CD4⁺ T cells might benefit the host by minimizing inflammation and tissue destruction (175). Epitope escape appears to play a limited role in the failure of CD4⁺ T cell responses (183, 184); this contrasts with the prominent role of epitope escape in the failure of CD8⁺ T cell responses, discussed below.

422

423 **6.3. CD8⁺ T cell failure**

HCV-specific CD8⁺ T cell responses fail due to T cell exhaustion and epitope escape. CD8⁺ T cell exhaustion occurs through a multi-step process in which antigen-specific cells progressively lose effector functions and reduce their expression of cytokine receptors needed for memory CD8⁺ T cell homeostatic proliferation (158). Exhaustion may be protective in persistent infections because it reduces the risk that

immune responses themselves will cause pathology (158). Exhausted, HCV antigen-specific CD8⁺ T cells can be observed in the blood and liver as acute infection progresses to chronicity (185-188). Features of these cells include expression of two or more inhibitory co-receptors (such as PD-1, 2B4, Tim-3, CTLA4, KLRG1, or CD160), and reduced expression of the IL-7 receptor α chain, CD127. IL-7, one of the common- γ chain receptor cytokine family members, supports memory CD8⁺ T cell survival, function, homeostatic and antigen-dependent proliferation (158, 189, 190). Other members of this cytokine family, IL-7, IL-15, and IL-21, also play key roles in T cell survival and function. Down-regulation of CD127 makes CD8⁺ T cells less responsive to IL-7. Extensive CD127 down-regulation during the acute phase predicts HCV persistence in experimentally infected chimpanzees (191). Of note, however, transient loss of CD127 also identifies effector T cells (192). As acute HCV infection progresses toward persistence, HCV-specific CD8⁺ T cells lose CD127 expression, gain PD-1 expression, and undergo caspase 9-mediated “death by neglect” due to loss of cytokine survival signals (193). In contrast, protective HCV-specific CD8⁺ T cells elicited by an experimental HCV vaccine expressed CD127 (194). Exhausted CD8⁺ T cells may be rescued *in vitro* with IL-2, IL-7 and IL-15 supplementation (193). IL-21 production by CD4⁺ T cells is associated with resolution of acute HCV infection (182).

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The association of exhausted HCV-specific T cells with chronic HCV infection raises the possibility of therapeutic efforts to reverse T cell exhaustion during chronic infection (171). Blockade of PD-1 during persistent LCMV infection in mice can restore immune-mediated control; however, PD-1 signaling also protects the body from immune-mediated pathology (195-197). Also important, intrahepatic T cells typically express at least some inhibitory receptors even in the absence of viral infection (188), and HCV-specific T cells continue to express PD-1 even after spontaneous clearance of infection (198). PD-1 blockade may not be sufficient to reverse T cell exhaustion (199) or restore immune control of HCV in an established persistent infection (200). Blockade of other inhibitory co-receptors, such as Tim-3 (179, 182), CTLA-4 (201), and/or 2B4 (188) might also hold some immunotherapeutic promise. The success of any therapeutic strategy aimed at blockade of inhibitory co-receptors would, of course, be contingent on the presence and survival of some critical number of antigen-specific T cells.

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6.4. Immune escape and T cell responses

Another pathway to the failure of HCV-specific T cells is immunological escape. HCV's error-prone replication mechanism produces amino acid changes in viral proteins at a dizzying rate (202). The resulting pool of novel viral RNA sequences provides the raw material for natural selection and rapid evolution under pressures that include immune recognition. HCV must evolve within each new host to maximize its potential to replicate and minimize immune-mediated clearance (203-209). As HCV infects a new host, viral sequence diversity may initially be limited by a founder effect. Those viral sequences capable of robust replication in the new environment have a selective advantage; sequence changes conferring efficient viral spread and replication sometimes restore consensus polypeptide sequences for a given HCV genotype (210). This may also represent a release of immunological pressure from the previous (donor) host. HCV may replicate for several weeks before the acutely infected host mounts an adaptive immune response. The onset of HCV-specific T cell responses is associated with signs of hepatocyte damage and a decline in viral load, suggesting that initial T cell responses mediate selection for viral sequence changes that abolish T cell recognition (211, 212), just as antibodies mediate selection for viral sequences that abolish antibody recognition (213, 214). The impact of T cell-mediated selection is seen in the selection of specific mutations in individuals expressing different HLA alleles (50, 167, 204, 215-220). For unknown reasons, selection mediated by HLA-B alleles may be stronger than that mediated by HLA-A alleles (167). Viral immune escape may be more successful in individuals mounting a less diverse T cell response (221, 222). Sequence changes are not unlimited, however, and viral immune escape is constrained by the need to retain replicative fitness (207, 208, 223). Some viral sequence changes that could mediate immunological escape are not tolerated because they reduce viral replicative fitness; T cell responses targeting these epitopes may be blunted by exhaustion and deletion (224, 225), as discussed in the previous paragraph. Once chronic infection is established, the selective pressure for continued T cell epitope escape is apparently lost (226-228). Reductions in immune pressure, for example during pregnancy, may be associated with reversion of escape mutations (223). Conversely, some exhausted T cells may be released from their exhausted state by loss of the epitope they target: viral sequence mutations that eliminate recognition by an exhausted T cell clone are associated with functional recovery of those T cells (229).

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7. The humoral immune response

Antibody (**Ab**) responses are observed in chronic HCV infection, but their roles in control of HCV remain controversial (230, 231). As observed for T cell responses, Ab responses are delayed during acute HCV infection compared to other viral infections (232, 233) (**Figure 4**). Clearance of acute HCV infection can occur in the absence of a detectable HCV-stimulated Ab response (166, 234-239), and Abs to HCV can wane or vanish after spontaneous or treatment-induced resolution of infection (33, 170, 240, 241). Ab responses target both structural and non-structural proteins. However, all known virus-neutralizing Ab (**nAb**) target the envelope glycoproteins E1 and, especially, E2. It is not clear whether Abs against non-structural proteins can recognize intact infected cells or affect HCV replication in infected cells; these Abs might contribute to clearance and opsonization of debris from infected tissue.

Lacking robust in vitro systems to study primary HCV isolates, researchers have turned to artificial in vitro systems to address questions of Ab-mediated neutralization of HCV infection (reviewed in (242)). Many recent studies have utilized lentiviral particles pseudotyped with HCV E1E2 heterodimers (**HCVpp**). HCVpp deliver reporter genes to hepatoma cell lines that express the HCV entry factors, SR-BI, CD81, Claudin-1 and Occludin (43, 242). Other studies have used chimeric cell-culture-derived HCV (**HCVcc**), substituting the core-NS2 regions (**Figure 1**) from HCV isolates of interest into the laboratory workhorse JFH-1 isolate (reviewed in (242)). Sera from patients with persistent HCV infection typically contain high-titer Abs able to neutralize infection by HCVpp bearing E1E2 from at least some genotypes (231, 237, 243, 244). Of interest, nAbs that block infection by HCVpp may be poorly effective against HCVcc (245).

Numerous studies support a role for nAbs in vivo. In chimpanzees, the closest animal model for human infection, early treatment with nAbs could prevent infection, and nAb infusion reduced viral load in persistent infection (246, 247). In immunodeficient mice with chimeric human livers, HCV infection can be prevented by nAb treatment before infection (248, 249), and persistent infection can be treated with nAb to induce an apparent cure (250). Similarly, HCV can infect the liver in mice expressing human CD81 and Occludin, and in these mice, too, broadly neutralizing monoclonal Abs can provide protection if

511 administered before infection (251, 252) and even after establishment of infection (250). Importantly, the
512 mouse models of HCV persistence used in these studies are, by necessity, immunodeficient, highlighting the
513 important role played by nAb.

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515 Humans, too, benefit from HCV-specific nAbs. Acutely infected patients destined to clear infection without
516 treatment may develop an early, broadly crossreactive (effective against multiple HCV genotypes) nAb
517 response, while early nAb responses are weaker and less broadly crossreactive in patients who progress to
518 chronic infection (33, 170, 241, 253-255). A rare incidence of spontaneous clearance of chronic infection
519 was reported to follow development of a broadly-reactive nAb response (170). Many broadly crossreactive
520 nAbs target a restricted set of epitopes within E2, and are believed to block viral binding to entry factors
521 (reviewed in (230, 231)). Broadly crossreactive nAbs may limit viral escape options, much as a broadly
522 directed T cell response does, by targeting highly conserved epitopes. However, a discouraging report
523 recently showed that nAb binding to highly conserved neutralizing epitopes is vulnerable to amino acid
524 changes in E2 at sites outside of the nAb epitope (214). Some amino acid changes of this type may have a
525 negative impact on viral infectivity (256). nAb may reduce viral loads sufficiently to release T cells from
526 exhaustion caused by excessive antigenic stimulation (170). nAb can provide passive protection against
527 infection and, indeed, nAb activity in bulk human immunoglobulin preparations protected recipients from
528 HCV infection before the advent of HCV serological screening (249, 257-259). Most serum nAb activity in
529 persistently infected individuals rests in an IgG fraction (237). The loss of CD4⁺ T cells in HIV co-infection
530 reduces HCV-specific nAb breadth and titers (260).

531

532 **7.1. Escape from nAb**

533 HCV is thought to escape from sterilizing humoral immunity by rapid sequence variation, by stimulating the
534 production of interfering antibodies, masking neutralization epitopes, and likely by concealing itself within
535 lipoviral particles (reviewed in (230, 231)). Competition between nAb and interfering non-neutralizing Ab
536 may disrupt nAb function: the CD81-binding loop region of E2 is targeted by both neutralizing and non-
537 neutralizing Abs, with some reports suggesting that the non-neutralizing Abs may limit nAb access to key
538 neutralization epitopes (261-264). This hypothesis is not universally accepted (231, 263-268). Neutralization

539 epitopes may be masked by extensive glycosylation (see below) (269). HCV is assembled into lipoviral
540 particles (42), and ultrastructural studies of HCVcc showed greater surface exposure of host apolipoprotein E
541 than of viral E2 (270). While it is thought that lipoviral particle association may protect HCV from nAb, it is
542 difficult to test this hypothesis quantitatively. Some HCV spread occurs between adjacent cells in a fashion
543 that is resistant to E2-specific nAb (46, 47, 271-275). This type of spread may remain sensitive to smaller
544 Ab-like reagents including an alpaca nanobody (276). Despite the multitude of potential escape mechanism,
545 a recent report showed nAb-dependent cure of persistent HCV infection in immunodeficient mice bearing
546 human liver grafts (250).

547

548 Structural and functional studies of E2 have revealed that key neutralization epitopes may be concealed
549 behind a decoy structure—the so called first hypervariable (**HVR1**) sequence near the amino terminus of E2
550 (259, 277, 278). This sequence is highly immunogenic but elicits weakly cross-reactive, isolate-specific Abs.
551 The HVR1 sequence is under few functional constraints, and indeed E2 lacking HVR1 is functional (278).
552 HCV readily tolerates amino acid changes that abolish recognition by HVR1-specific Abs. Several reports
553 link E2 sequence evolution to nAb escape during chronic infection (213, 254, 279, 280). Host nAb responses
554 lag behind the shape-shifting E2 (213, 254). That nAbs fail to neutralise the dominant viral strain at a given
555 time, yet successfully neutralise previously dominant viral strains in the same patient, demonstrates the
556 continued evolution and escape of the virus under selective pressure from nAbs, with the humoral immune
557 system always, sadly, one step behind (213). Whereas nAb responses select E1E2 sequence variation over
558 time, envelope sequence changes are not observed in hypogammaglobulinemic patients (281-283). nAb with
559 broad, multi-isolate reactivity bind to highly conserved and functionally constrained sequences involved in
560 entry factor binding. These epitopes are protected by the HVR (259, 277, 278, 284) and, as discussed in the
561 next paragraph, by glycosylation.

562

563 The ectodomains of HCV's E1 and E2 envelope glycoproteins are heavily glycosylated: glycans contribute
564 almost half of the ectodomains' apparent molecular weight. E2, which is more immunogenic than E1,
565 contains nine N-linked glycosylation sites conserved across all HCV genotypes (two additional sites are
566 conserved in most isolates). Glycans play essential roles in assembly and folding of the E1E2 heterodimer,

567 and are required for viral entry (269, 285-287). The glycans limit nAb access to E1E2, protecting virus from
568 neutralization (269). Structural analysis of E2-nAb complexes showed that heavy glycosylation can mask
569 neutralization epitopes (284). Removal of the glycan shield increases HCVpp sensitivity to nAb (288).

570

571 **7.2 An immune response gone wrong? Mixed cryoglobulinemia**

572 Hepatocytes are the primary target of HCV infection, but as discussed elsewhere in this book, B lymphocyte
573 dysfunction and malignancy lead to extrahepatic disease in many chronic HCV patients (289, 290). Mixed
574 cryoglobulinemia (MC) is a common extrahepatic manifestation (reviewed in (291, 292)). In MC, the
575 intravascular deposition of immune complexes, containing IgM rheumatoid factor, polyclonal IgG, and viral
576 RNA, elicits an inflammatory reaction that can lead to vasculitis, nephropathy, and neuropathy (291, 292);
577 this apparently benign lymphoproliferative condition may progress to B cell non-Hodgkin lymphoma (293).
578 Successful antiviral therapy may result in complete regression of HCV-associated lymphoma (293). B cells
579 expressing rheumatoid factor-like IgM are clonally expanded in HCV patients with symptomatic MC,
580 supporting the hypothesis that MC arises through antigen-driven stimulation of specific B cell clones (294,
581 295). Notably, many reports indicate that clonally-expanded B cells in HCV-associated MC, as well as
582 HCV-associated non-Hodgkin lymphomas, express a stereotypical antigen receptor encoded by rearranged
583 VH1-69 (also called VH51p1) and Vκ3-20 (also known as kv325 and A27) variable region genes (294, 296-
584 301).

585

586 Other mechanisms have been proposed for the development of HCV-associated MC and non-Hodgkin
587 lymphoma, but these mechanisms are not supported by our current understanding of HCV tropism, structure,
588 and replication (reviewed in (233)). Models proposing that HCV's E2 envelope protein induces polyclonal B
589 cell activation by crosslinking CD81 on B cells (302) are inconsistent with clinical observations, in that B
590 cell activation in MC is clonally restricted rather than polyclonal (294, 296-301). Furthermore, it has not
591 been demonstrated that intact circulating lipoviral particles (42) can mediate the activation reported in vitro
592 with high concentrations of recombinant E2. While it has been suggested that HCV may infect B cells (303),
593 and thereby cause mutations or functional changes, B cells do not express the array of entry factors
594 (including two tight junction proteins, Claudin-1 and Occludin) needed for HCV infection of hepatocytes

595 (42, 44, 233). Neither cell culture-derived HCV of genotype 2a, nor HCV pseudoparticles bearing envelope
596 glycoproteins of various genotypes, can infect primary B cells or B cell lines (44, 304). Levels of HCV RNA
597 associated with patient lymphocytes are far below one copy per cell (140, 305-307), suggesting that
598 replication, if any, is inefficient in human lymphocytes. Observations of similar clonally expanded B cells in
599 multiple MC patients, in different studies from different researchers, strongly suggest that a common
600 antigenic stimulus plays a role in development of MC. Work is ongoing to probe the roles of viral and self
601 antigens in driving typical MC-related clonal expansion (308); host genetic factors (309) and changes in
602 cytokine levels (310) may also contribute.

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604 **8. Outlook**

605 As we move into an era in which diagnosed HCV infections can be treated effectively (although not
606 economically), a prophylactic vaccine remains a critical unmet need (21, 22, 311, 312). Therapeutic vaccines
607 and immune enhancement strategies may also contribute to future treatment regimes. Understanding the
608 mechanisms of immune control and failure will be essential to the development of effective vaccines.
609 Significant progress has been made (22). Roles for both humoral and cell-mediated immunity must be
610 considered. A prophylactic vaccine stimulating a robust, broadly-directed (30, 313), cross-reactive (multiple
611 genotypes) (314), and polyfunctional (194, 313) T cell response is desirable if the goal is to prevent
612 persistent HCV infection. This will be challenging because, even in subjects who have achieved spontaneous
613 resolution of HCV infection, immunity may be largely strain or genotype-specific. Viral epitopes that can
614 stimulate a universally protective anti-HCV immune response have not yet been identified. A prophylactic
615 vaccine that stimulates production of broadly reactive nAb is appealing because of its potential to block even
616 acute HCV infection. Emerging evidence indicates that broadly crossreactive nAb can treat established
617 infection (250), underscoring the need for inclusion of the structural proteins, E1 and E2, in vaccine
618 candidates.

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1410

1411 **Figure legends**

1412 **Figure 1.** HCV RNA and polyprotein. Conserved RNA structures in the 5' and 3' untranslated regions
1413 (UTR) are illustrated. The genome has a single long reading frame encoding a viral polyprotein of
1414 approximately 3000 amino acids. The polyprotein is processed co- and post-translationally by host and viral
1415 proteases to release the three structural proteins, Core, E1, and E2 (labeled in blue) and the seven non-
1416 structural proteins (labeled in red).

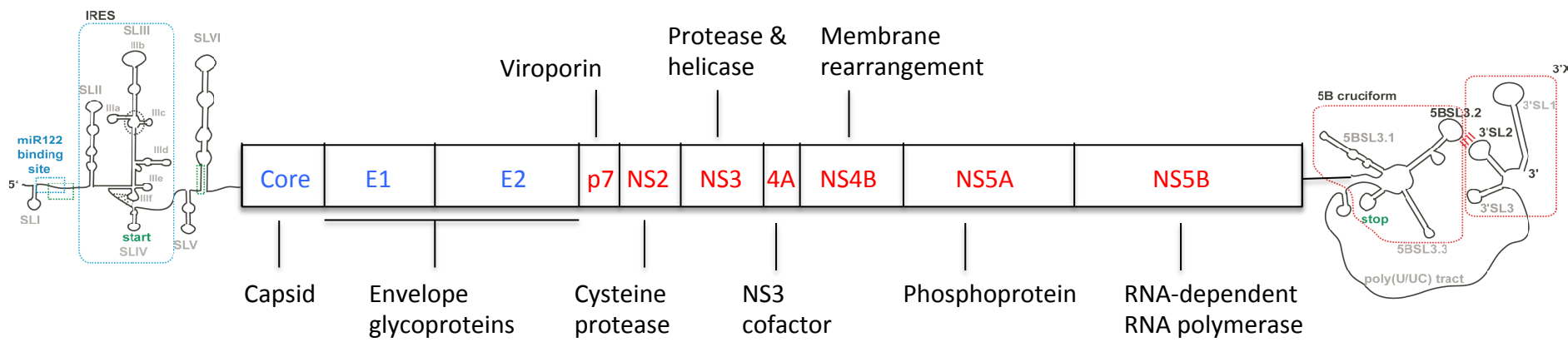
1417
1418 **Figure 2.** Innate immune activation by HCV RNA. A) RIG-I binds to PAMPs in HCV RNA and changes its
1419 conformation, activating one or more E3 ubiquitin ligases (yellow shapes). Ubiquitinated RIG-I may activate
1420 MAVS, which then forms prion-like aggregates that recruit additional ubiquitin ligases. Recent evidence
1421 suggests that MAVS on peroxisomes is particularly important for induction of IFN λ . Subsequent
1422 ubiquitination steps stimulate recruitment of enzymes that activate the downstream IRF3 and NF κ B
1423 pathways. HCV's NS3/4A protease can disable this pathway by cleaving MAVS near its transmembrane
1424 domain. B) TLR3 recognizes dsRNA within endosomal compartments, signaling via the adaptor, TRIF.
1425 TRIF stimulates ubiquitin-conjugating enzymes, resulting in recruitment of enzymes that activate the IRF3
1426 and NF κ B transcription factors. HCV's NS3-4A protease can disable TLR3 signaling by cleaving TRIF.

1427
1428 **Figure 3.** T cell responses in acute resolving (A-B) and chronic (C-D) HCV infection. A) Patterns of viremia
1429 (HCV RNA) and liver cell death (alanine aminotransferase, ALT, which is released from damaged
1430 hepatocytes) in spontaneous resolution of HCV infection. B) CD4⁺ and CD8⁺ T cells respond to multiple
1431 HCV epitopes until viremia is cleared and afterwards. C) Patterns of viremia and liver cell death in chronic
1432 infection. D) CD4⁺ and CD8⁺ T cell responses wane as viremia persists. CD8⁺ T cells lose function due to
1433 exhaustion after loss of CD4⁺ T cell help. CD8⁺ T cells also select for variant virus sequences that escape
1434 immune detection.

1435
1436 **Figure 4.** Humoral immune responses in acute resolving (A) and chronic (B) HCV infection. A) Rapid
1437 development of HCV-specific nAb may contribute to spontaneous resolution of infection. Ab to HCV
1438 structural and non-structural proteins can be detected by enzyme-linked immunoassay (EIA). Ab levels may

1439 decline after infection is cleared. B) Slower development of nAb responses may predispose to chronic
1440 infection. Ab to structural and non-structural proteins is detectable by EIA.
1441
1442
1443

5' UTR Structural proteins Non-structural proteins 3' UTR



A

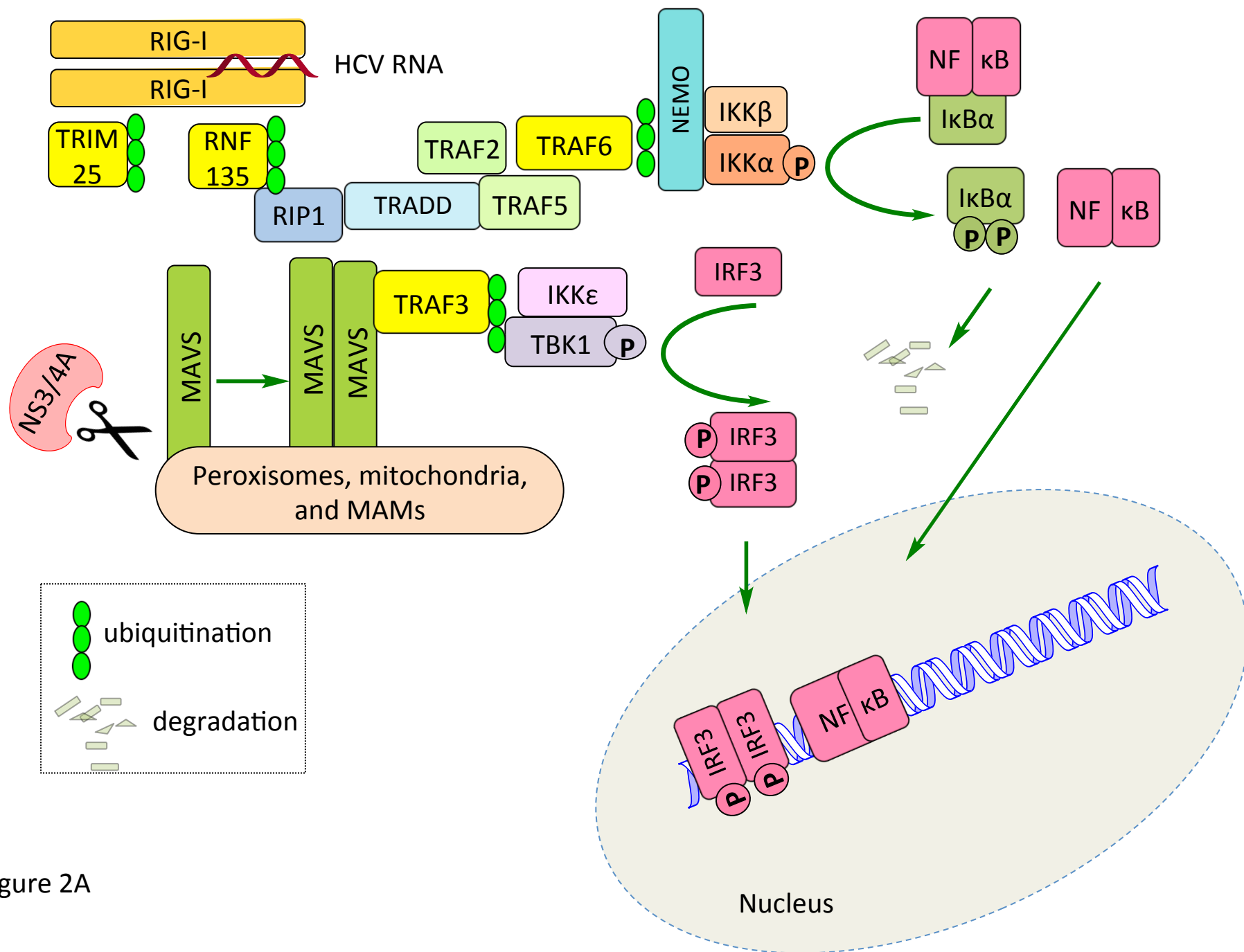


Figure 2A

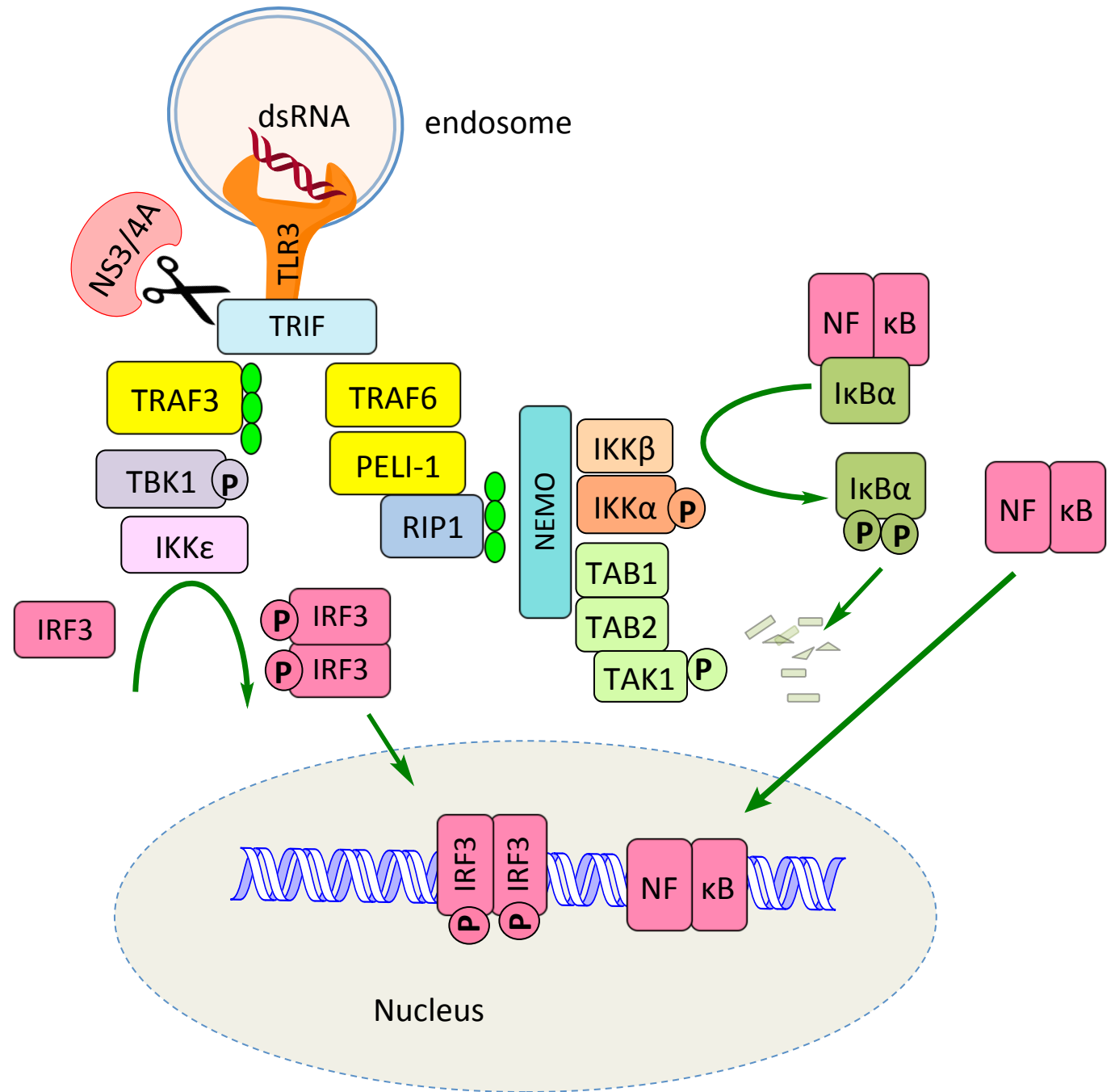
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Figure 2B

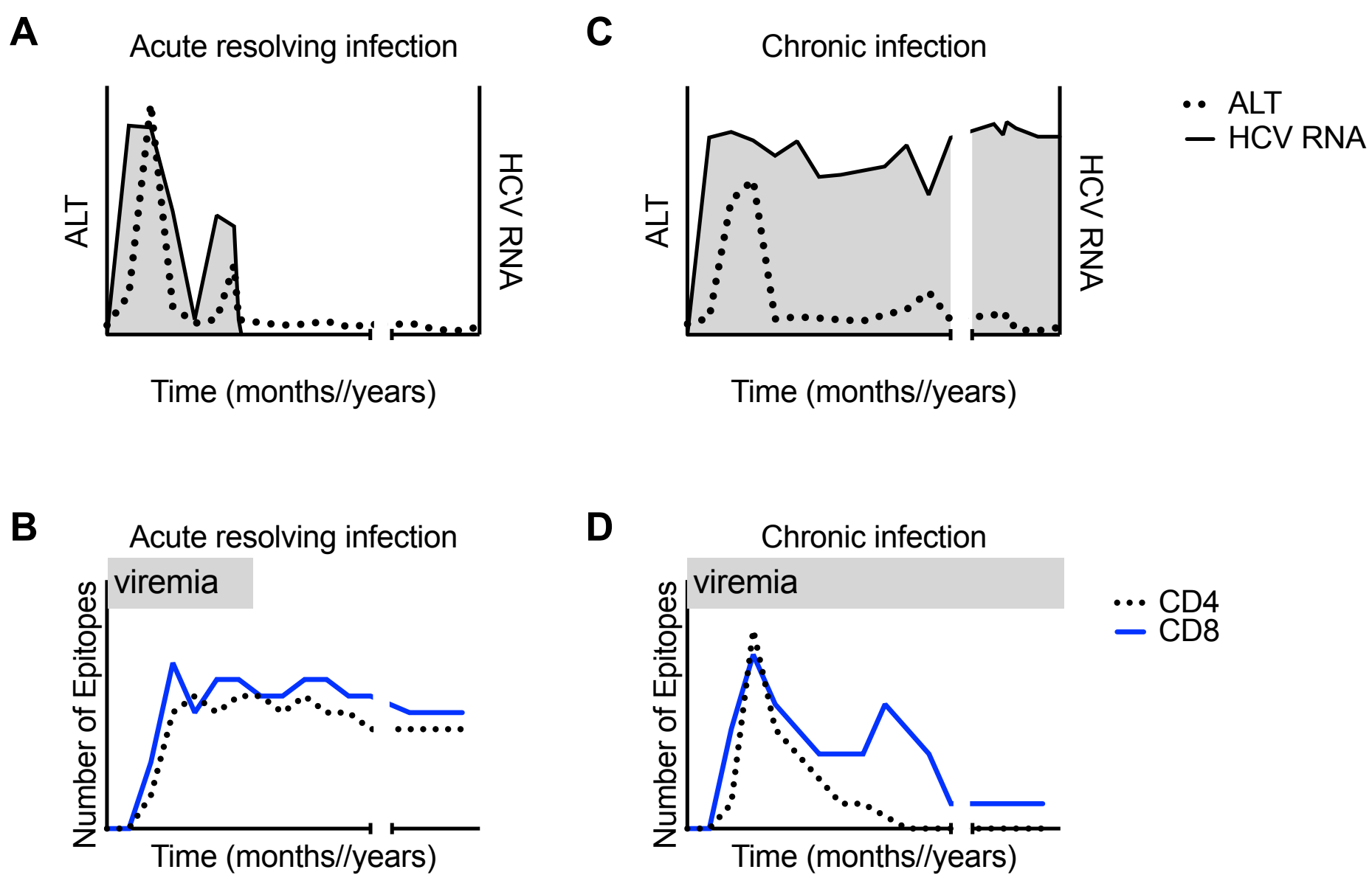
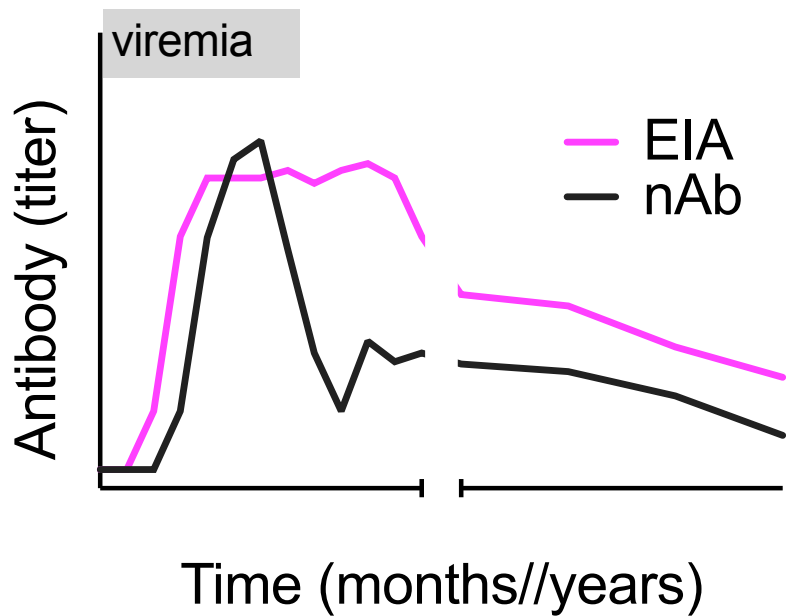


Figure 3

A Acute resolving infection



B Chronic infection

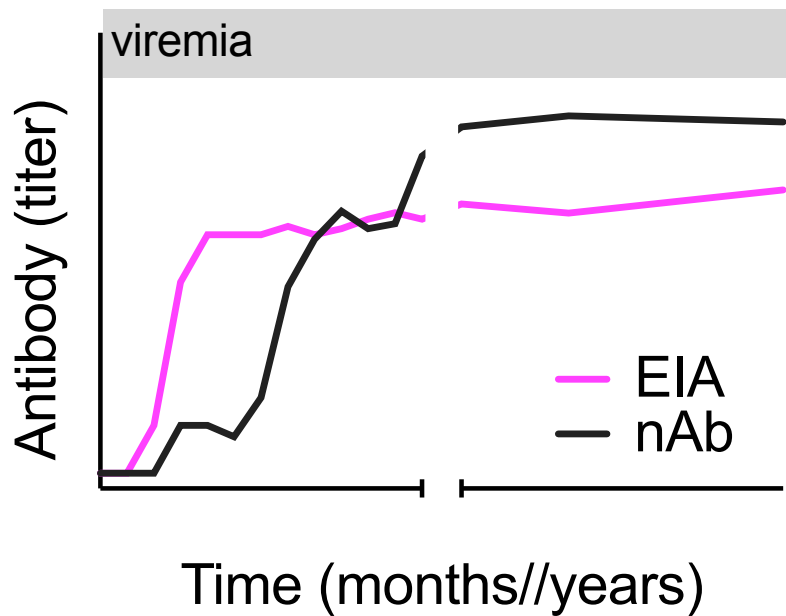


Figure 4