1   **Viral vectored hepatitis C virus vaccines generate pan-genotypic T cell responses to**

2   **conserved subdominant epitopes**

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HIGHLIGHTS:

1. Conserved segment HCV vaccines induce high magnitude CD4$^+$ and CD8$^+$ T cell responses in mice.

2. Conserved segment HCV vaccines are as immunogenic as the gt1b HCV vaccine that was in human trials.

3. Conserved segment HCV vaccine induced T cells target highly conserved epitopes across subtypes.

4. These highly conserved epitopes are associated with spontaneous HCV resolution in humans.

5. Adding the truncated shark invariant chain to the HCV immunogen increases the T cell response.

ABSTRACT

Background: Viral genetic variability presents a major challenge to the development of a prophylactic hepatitis C virus (HCV) vaccine. A promising HCV vaccine using chimpanzee adenoviral vectors (ChAd) encoding a genotype (gt) 1b non-structural protein (ChAd-Gt1b-NS) generated high magnitude T cell responses. However, these T cells showed reduced cross-recognition of dominant epitope variants and the vaccine has recently been shown to be ineffective at preventing chronic HCV. To address the challenge of viral diversity, we developed ChAd vaccines encoding HCV genomic sequences that are conserved between all major HCV genotypes and adjuvanted by truncated shark invariant chain (sII$_{tr}$).

Methods: Age-matched female mice were immunised intramuscularly with ChAd (10^8 infectious units) encoding gt-1 and -3 (ChAd-Gt1/3) or gt-1 to 6 (ChAd-Gt1-6) conserved segments spanning the HCV proteome, or gt-1b (ChAd-Gt1b-NS control), with immunogenicity assessed 14-days post-vaccination.

Results: Conserved segment vaccines, ChAd-Gt1/3 and ChAd-Gt1-6, generated high-magnitude, broad, and functional CD4$^+$ and CD8$^+$ T cell responses. Compared to the ChAd-Gt1b-NS vaccine, these vaccines generated significantly greater responses against conserved non-gt-1 antigens, including conserved subdominant epitopes that were not targeted by ChAd-Gt1b-NS. Epitopes targeted by the conserved segment HCV vaccine induced T cells, displayed 96.6% mean sequence homology between all HCV subtypes (100% sequence homology for the majority of genotype-1, -2, -4 sequences and 94% sequence homology for gt-3, -6, -7, and -8) in contrast to 85.1% mean sequence homology for epitopes targeted by ChAd-Gt1b-NS induced T cells. The addition of truncated shark invariant chain (sII$_{tr}$) increased the magnitude, breadth, and cross-reactivity of the T cell response.

Conclusions: We have demonstrated that genetically adjuvanted ChAd vectored HCV T cell vaccines encoding genetic sequences conserved between genotypes are immunogenic, activating T cells that target subdominant conserved HCV epitopes. These pre-clinical studies support the use of conserved segment HCV T cell vaccines in human clinical trials.

Keywords: Universal HCV vaccine, adenovirus, conserved sequence, cross-reactive, invariant chain

INTRODUCTION

With approximately 71 million worldwide infections and 400,000 deaths annually, hepatitis C virus (HCV) remains a major cause of liver disease and liver cancer globally (1). Despite the advent of highly-effective directly acting anti-viral drugs (DAAs) to treat HCV-infected individuals (2), the WHO recently reported that the rate of new HCV infections (1.75 million annually) exceeds the number of people dying of HCV or enrolled on HCV treatment programmes (1). This is partly due to approximately 80% of HCV infections being asymptomatic resulting in low treatment rates and underdiagnosis. In some settings the transmission of drug resistant HCV variants to new people has been reported (3). Even after sustained virologic response (SVR), patients with cirrhotic livers are still at risk of developing liver cancer after HCV clearance (4), and all DAA-resolved patients remain vulnerable to HCV re-infection, a significant problem for high-risk populations (5). Finally, treatment enrolment rates remain low due to relatively high drug costs, particularly in low-middle income countries (LMICs) with limited health resources (1). Therefore, there remains an urgent need to develop a prophylactic HCV vaccine, in addition to the current strategy of treating patients with DAAs that present with clinical infection (6).

A prophylactic HCV vaccine should be an attainable goal since 20% of HCV-infected individuals spontaneously resolve acute infection (1) associated with the generation of HCV-specific T cells targeting a broad range of HCV antigens (7–15). Neutralising antibodies may also play an important role in resolving infection but when generated these appear to be largely strain specific (16–18). A wealth of evidence shows that T cell immunity is causally linked to viral control, including the fact that spontaneous resolution increases from ~25% in primary infection to ~85% with a rapid T cell memory recall response following secondary HCV exposure (18), an association of viral clearance with class I and II human leukocyte antigens (HLA; HLA-A3, HLA-B27, HLA-B57, HLA-DR1101, and HLA-DQ0301 antigens (19–22) and the observation that antibody-mediated depletion of CD4^{+} and CD8^{+} T cells leads to viral persistence in HCV challenged chimpanzees (23,24) and rat hepacivirus (RHV) challenged vaccinated rats (25). A successful HCV T cell vaccine should seek to mimic the effective immune response that has been demonstrated in natural infection but should also provide broad coverage against common viral genotypes.

4

94 Very recently, preliminary results from a phase II study (ClinicalTrials.gov NCT01436357) evaluating a

95 promising HCV T cell vaccine strategy in people who inject drugs (PWIDS) have been reported

96 (www.niaid.nih.gov/news-events/trial-evaluating-experimental-hepatitis-c-vaccine-concludes). This

97 approach used chimpanzee adenovirus and modified vaccinia Ankara (ChAd3 and MVA) viral vectors

98 encoding the gt-1b specific sequence of non-structural (NS) proteins 3-5 (1985 amino acids), in a

99 heterologous prime/boost strategy. These vectors, when used in prime/boost have been shown to be

100 potent inducers of cellular immune responses against the encoded immunogen, in part due to an

101 intrinsic adjuvant effect of the vectors. In spite of the high magnitude of polyfunctional CD4$^+$ and CD8$^+$

102 generated by this approach as demonstrated in early phase I human trials (26), this vaccine failed to

103 protect PWIDS from chronic infection (27). Whilst the data indicating why this vaccine trial failed to

104 protect people from chronic HCV infection is yet to be reported, the lack of protection highlights the

105 need for alternative vaccine strategies.

106

107 HCV viral variability has long been recognised as a major challenge to the development of an HCV

108 vaccine, with six common distinct HCV genotypes that are 20% divergent at the amino acid level and

109 over one hundred genetically different subtypes worldwide (28). Although our previous data

110 evaluating the gt-1b vaccine (reported in NCT01436357) showed evidence of T cell immune

111 responses that were cross reactive with non-gt 1b antigens, these were reduced by more than 50%

112 (26). Furthermore, when evaluating HCV specific T cell responses at the single epitope level we found

113 that there was a marked reduction or absence of T cell responses against commonly circulating

114 epitope variants both within and between HCV genotypes (29). In patients exposed to HCV, we have

115 also shown limited cross reactivity between T cells that target gt-1 and gt-3 (30) which are the two

116 dominant HCV genotypes globally (31). This lack of T cell cross reactivity in dominant epitopes is

117 likely to present a major challenge to real world scenarios where multiple HCV genotypes are found

118 circulating within the same geographical regions, and where the virus population within a host (the

119 quasispecies) exhibits genetic variation that may rapidly escape the immune response (32). An

120 effective vaccination strategy will need to target multiple genotypes within a target population and

121 virus variants within an infected individual in order to overcome HCV variability and prevent viral

122 persistence.

123

124 We therefore generated second generation HCV T cell immunogens (33), encoding conserved

125 genomic sequence between genotype-1 and -3 (gt1/3) aiming to provide coverage for the two most

126 dominant strains in Europe, and genotype-1, -2, -3, -4, -5 and -6 inclusive (gt1-6) to provide global

127 coverage against all major genotypes (31). These conserved sequence immunogens consist of

128 multiple segments of highly conserved HCV sequence across all HCV subtypes, and exclude variable

129 HCV regions (33). We hypothesised that this approach would generate pan-genotypic T cell

130 responses and also limit viral escape from vaccine-induced T cell immunity since mutations within

131 conserved viral sequences are likely to carry a detrimental fitness cost (33). The gt-1-6 vaccine is

132 particularly attractive as a global vaccine as it would best mitigate against infections from a broad

133 range of genotypes in the current era of extensive travel and migration, reduce the risk of vaccine

134 escape mutations, and is most attractive from a manufacturing and commercial perspective as it

135 would focus clinical development on a single vaccine. Having previously described the rationale,

136 development, and generation of the conserved segment vaccine candidates (ChAd-Gt1/3 and ChAd-

137 Gt1-6; 32) we now evaluate the T cell cross-reactivity against dominant genotypes of these second

138 generation vaccines in comparison to ChAd-Gt1b-NS. We also aim to enhance T cell immune

139 responses against conserved regions of the HCV proteome using the truncated form of the shark

140 invariant chain (sIi$_{tr}$) previously shown to enhance T cell immune responses in malaria vaccine pre-

141 clinical studies (34).

142

143

144 **RESULTS**

145 **Conserved segment HCV immunogens induce high-magnitude T cell responses in mice**

146 Second generation HCV T cell vaccines encoding either long (1500 amino acid immunogen; L) and

147 short (1000 amino acid immunogen; S) HCV genomic segments conserved between (i) HCV

148 genotypes -1 and -3 (ChAd-Gt1/3), and (ii) genotypes-1 to -6 (ChAd-Gt1-6) (**figure 1A**) were

149 encoded in simian adenovirus vectors as previously described (ChAdOx1; 32). These vaccines (given

150 intramuscular (IM) at 10^8 infectious units; IU) generated high-magnitude IFNγ producing T cell

151 response as measured in splenocytes from vaccinated *BALB/c* mice harvested 14-days post-

152 immunisation (**figure 1B-C**). The long versions of the conserved segment immunogens (Gt1/3L and

153 Gt1-6L), that contain all *in silico* defined conserved sequences, displayed significantly higher median

154   frequencies of T cells of 2390 and 2455 median spot forming unit (SFU), respectively, in *ex vivo* IFNγ

155   ELISpot assays, compared to their respective short versions (p = 0.0286; **figure 1C**). The enhanced T

156   cell response with the long version immunogen was seen across multiple antigenic genomic regions

157   (assessed in 10 peptide pools) (**figure 1D**), predominantly targeting non-structural antigens NS3h,

158   NS4, and NS5b.

159

160   To limit the potential immunogenicity of artificial newly formed epitopes between genomic segments

161   that are not naturally occurring and are therefore irrelevant, short linker sequences of glycine, proline,

162   serine, and lysine residue combinations were inserted between segments that contain *in silico*

163   predicted strong binding epitopes (**suppl. figure 1A**). We constructed a Gt1-6L vaccine without linker

164   sequences and showed that this generated a significantly reduced T cell response in mice compared

165   to the Gt1-6L immunogen with linkers (p = 0.0286; **suppl. figure 1B-D**). We found no evidence that

166   the T cell response generated by the vaccine was directed to the linker regions (**suppl. figure 1E**).

167

168   **Conserved segment vaccines induce higher magnitude T cell responses than the ChAd-Gt1b-**

169   **NS vaccine**

170   Immunogenicity of conserved segment vaccines (ChAd-Gt1/3L and ChAd-Gt1-6L) were compared

171   with ChAd-Gt1b-NS, a vaccine containing the full length non-structural (NS3-5) region of a genotype

172   1b strain (BK strain) in *BALB/c* mice (**figure 2A**). At 14-days post-immunisation, conserved segment

173   HCV vaccines induced significantly higher frequencies of IFNγ^+ T cells with 2653 and 2330 median

174   SFU/10^6 splenocytes for ChAd-Gt1/3L (p = 0.0422) and ChAd-Gt1-6L (p = 0.0294), respectively,

175   when compared to ChAd-Gt1b-NS induced IFNγ^+ T cell frequencies (699 median SFU/10^6

176   splenocytes; **figure 2B**). The breadth of the immune response generated by the conserved segment

177   vaccines was similar or higher than that induced by the ChAd-Gt1b-NS vaccine and targeted both NS

178   and structural proteins (**figure 2C and 2D**). Vaccine-induced T cell responses were also assessed in

179   transgenic *HLA-A*02:01* transgenic mice (**figure 2E**); with the ChAd-Gt1b-NS generating T cells that

180   predominantly targeted the epitope, NS3$_{1585-1593}$, whilst the conserved segment vaccines

181   predominantly targeted the E2$_{614-622}$ epitope suggesting that the composition of the vaccine

182   immunogen may influence the hierarchy of T cell immune responses (immunogenic epitopes in

183   **suppl. table 1**).

184

Conserved segment vaccines induce inter-genotypic cross-reactive T cell responses

To assess inter-genotypic T cell responses generated by vaccination, we stimulated splenocytes *ex vivo* with HCV peptide pools specific for three genotypes/subtypes: -1a (H77), -1b (J4), and -3a (k3a650). Conserved segment HCV T cell vaccines (ChAd-Gt1/3L and ChAd-Gt1-6L) induced high-magnitude T cell responses to genotypes-1a (1432 and 994 median SFU/10^6 splenocytes), -1b (2390 and 2486 median SFU/10^6 splenocytes), and -3a (2609 and 1864 median SFU/10^6 splenocytes, respectively; **figure 3A)**. Overall, the conserved segment vaccines generated comparable HCV specific immune responses to each HCV genotype compared to the ChAd-Gt1b-NS vaccine, but most notably conserved segment vaccines generated significantly higher frequencies of IFNγ T cells specific for subtype-1b (Gt1-6L 2486 vs Gt1b-NS 664 median SFU/10^6 splenocytes; $p < 0.0001$) and -3a compared to ChAd-Gt1b-NS (1863 vs 588 median SFU/10^6 splenocytes, respectively; $p < 0001$; **figure 3A**). Significantly broader T cell immune responses were also generated, particularly to gt-1a/1b antigens with the ChAd-Gt1-6L vaccine ($p = 0.0476$) and gt-3a antigens with the ChAd-Gt1/3L vaccine ($p < 0.0001$; **figure 3B**). For all HCV vaccines, the genotype-1b and -3a ELISpot responses positively correlated (**figure 3C**).

Conserved segment HCV T cell vaccine induces plurifunctional CD4$^+$ and CD8$^+$ T cells

The functionality of vaccine-induced T cell response was determined using intracellular cytokine staining (ICS) by flow cytometry (gating and FACS plots; **suppl. figure 2**) following vaccination with ChAd-Gt1-6L. CD8$^+$ T cells that produced IFNγ, TNFα, and IL-2 were readily detected in murine splenocytes two-weeks post-vaccination (**figure 4A**). CD4$^+$ T cells were also detected but at a lower frequency than CD8$^+$ T cells (**figure 4B**). Both CD4$^+$ and CD8$^+$ T cells stimulated by gt-1b and -3a peptides displayed plurifunctionality of at least two cytokines, with CD8$^+$ T cells secreting all three cytokines after a gt-1b stimulus whereas CD4$^+$ T cells secreted all three cytokines after a gt-3a stimulus (**figure 4C-D**).

Conserved segment HCV vaccine induced T cell responses targets highly conserved sub-dominant epitopes across HCV subtypes

Dominant T cell epitopes targeted by the ChAd-Gt1b-NS in human studies have been shown to display high sequence variability at the population level (29). We therefore investigated the specificity

and variability of epitopes targeted by the ChAd-Gt1-6L vaccine compared to ChAd-Gt1b-NS vaccine-induced T cells targeted, using the splenocytes from outbred *CD-1* mice *ex vivo* with peptide minipools and individual peptides that correspond to the genotype-1b NS proteome (outbred mice used for increased variation of H antigen to present diverse T cell epitopes to T cells). The ChAd-Gt1b-NS and ChAd-Gt1-6L induced T cells that targeted different epitopes, with the ChAd-Gt1b-NS targeting epitopes that are generally not found in conserved genomic regions (**figure 5A** and epitope mapping given in **suppl. figure 3**). Next we determined the degree of conservation of these epitopes across all known HCV subtypes (n=223) as listed by the International Committee for the Taxonomy of Viruses (https://talk.ictvonline.org/ictv_wikis/flaviviridae/w/sg_flavi/634/table-1---confirmed-hcv-genotypes-subtypes-may-2019). The ChAd-Gt1-6L generated T cells targeted epitopes that are generally greater than 90% conserved between and within all HCV subtypes (with $NS3_{1244-1260}$, $NS4b_{1766-1781}$, and $NSb_{2756-2773}$ > 96%), including the newly described genotype-7 and -8 strains that were not incorporated in the original vaccine design algorithm. Whereas, the ChAd-Gt1b-NS vaccine induced T cells targeted epitopes that were markedly less conserved (**figure 5B and suppl. figure 4**), an observation that was highly statistically significant between immunodominant epitopes for each vaccine ($NS3_{1634}$ for ChAd-Gt1b-NS and $NS4b_{1766}$ for ChAd-Gt1-6L) when comparing all epitope variants across genotype-1 to -8 (p < 0.0001; **figure 5C)**. The targeting of different epitopes by ChAd-Gt1-6L compared to ChAd-Gt1b-NS was replicated in *C57BL/6* inbred mice, by both CD8$^+$ and CD4$^+$ T cells (**suppl. figure 5**). All targeted epitopes found in mice have been previously described in human HCV infection (immunogenic epitopes in mice listed in **table 1**).

A novel genetic adjuvant—the transmembrane region of the shark invariant chain (sIi$_{tr}$)—increases vaccine-induced T cell response

As subdominant epitopes may generate lower magnitude T cell responses (due to a lower frequency of naïve T cell populations or through less efficient antigen presentation), increasing vaccine-induced T cell responses using genetic adjuvants may be a useful strategy. Full length and truncated Ii genetic adjuvants have recently been shown to enhance T cell responses between 2- and 5-fold (34,35)*. Therefore, we investigated the effect of encoding the novel genetic adjuvant, sIi$_{tr}$ (a truncated sequence from shark MHC class II invariant chain), at the 5' end of the conserved HCV sequence transgene within the ChAd viral vector, sIi$_{tr}$, shares 24.6% sequence homology with human Ii in a

244 truncated form (**figure 6A**), which was previously shown to enhance immune responses to encoded

245 antigens (34). The sIi$_{tr}$ adjuvanted vaccine demonstrated a significant increase in T cell magnitude

246 compared to non-adjuvanted vaccine (No GA), but not when compared to a vaccine adjuvanted using

247 the tissue plasminogen activator leading sequence (TPA-LS) that is also known to enhance T cell

248 immune responses (35; **figure 6B-C**). The sIi$_{tr}$ adjuvanted vaccine also induced significantly broader

249 HCV specific immune responses, targeting 19/24 conserved gt1-6L sequence segments, when

250 compared to 6/24 conserved gt1-6L sequence segments targeted by non-adjuvanted vaccines (p =

251 0.0086; **figure 6C-D**). The sIi$_{tr}$ adjuvanted ChAd-Gt1-6L vaccine also increased the HCV genotype-

252 1a, -1b, and -3a specific total IFNγ ELISpot response in *CD-1* outbred mice compared to the TPA-LS

253 adjuvanted gt1-6L vaccine (p = 0.0471; statistically significant for gt-1a and -1b peptide stimulation

254 **figure 6E**).

255

256 **DISCUSSION**

257 Recent efforts to generate a prophylactic vaccine against HCV have used viral vectors encoding a

258 genotype-1b immunogen (ChAd-Gt1b-NS), generating high magnitude, broad, polyfunctional T cells

259 when used in heterologous prime boost strategies, in healthy human volunteers (26,37). However, our

260 previous work has also shown that some T cell responses generated by this approach target

261 immunodominant epitopes with limited cross-reactivity to non-vaccine genotypes and a recent press

262 release by NIH has concluded that this vaccine was not effective in preventing chronic infection in at

263 risk PWID (27). We have therefore developed second generation HCV vaccines, ChAd-Gt1/3 and

264 ChAd-Gt1-6, encoding HCV genomic segments that are conserved between HCV subtypes encoded

265 in a ChAdOx1 viral vector (33) specifically designed to address the global coverage of different HCV

266 genotypes and assessed these in pre-clinical studies.

267

268 We show that ChAd-Gt1/3 and ChAd-Gt1-6 vaccines generate HCV specific T cell responses that are

269 of a higher magnitude than those induced by the ChAd-Gt1b-NS vaccine in inbred, outbred, and HLA-

270 A2.1 transgenic mice. These conserved segment vaccines were designed to induce T cells against

271 both structural and non-structural HCV antigens; in mice these T cells predominantly target non-

272 structural HCV antigens, though T cell responses to structural antigens were also detected at low

273 magnitude. Both CD4$^+$ and CD8$^+$ T cell subsets are generated from a single prime vaccination

10

274 secreting IFNγ, TNFα, and IL-2. The generation of both CD4$^+$ and CD8$^+$ T cells is an important

275 criterion for the selection of vaccine candidates for human studies, since HCV resolution has been

276 associated with the generation of CD4$^+$ and CD8$^+$ T cells that secrete these cytokines

277 (7,11,23,24,38,39).

278

279 Non-structural HCV epitopes targeted by ChAd-Gt1-6 induced T cells (NS3$_{1244-1260}$, NS4$_{1776-1781}$), but

280 not by ChAd-Gt1b-NS induced T cells, have been previously identified in the majority of acute

281 resolving gt-1a/b, -3a, and -4 HCV infections (7,12,30,40–42). In contrast, epitopes in non-structural

282 HCV sequence targeted by ChAd-Gt1b-NS induced T cells (NS3$_{1621-1637}$, NS5a$_{2278-2278}$, NS5b$_{2447-2470}$,

283 NS5b$_{2955-2972}$) have been described only in a minority (~18%; 40) of resolving HCV gt-1 and -3

284 infections (12). Furthermore, a structural HCV epitope previously described in spontaneous

285 resolution, E2$_{606-622}$ (7,12,30,43), was targeted by the ChAd-Gt1-6 induced CD4$^+$ T cell response

286 (HLA-A2 and CD-1 mice). Whilst the generation of E2 specific T cells may in theory contribute to the

287 generation of anti-HCV antibodies (through T cell help), this vaccine is not designed to generate

288 antibodies, and these were not evaluated. Overall, these observations from spontaneous resolvers

289 suggest that the induction of conserved subdominant epitopes may be preferable to combat multiple

290 HCV genotypes.

291

292 Immunodominant CD4$^+$ and CD8$^+$ T cell epitopes in variable viral regions display limited cross

293 reactivity between HCV genotypes (30,42,44,45). The exclusion of variable HCV sequences

294 containing immunodominant epitopes from an HCV immunogen may increase the targeting of

295 subdominant epitopes by naïve T cells and therefore generate a vaccine-induced T cell response

296 targeting subdominant epitopes that lie in conserved viral regions. Here, we demonstrate that the

297 conserved segment vaccines generate T cells that target highly conserved subdominant epitopes

298 (greater than 96.6% sequence homology across HCV subtypes) that are not targeted by the ChAd-

299 Gt1b-NS vaccine, whereas the ChAd-Gt1b-NS vaccine generates T cells that target immunodominant

300 epitopes that are not found in conserved viral regions. This result demonstrates that there is a

301 hierarchy of immune dominance that may be manipulated through the exclusion or inclusion of

302 particular genomic regions in rational vaccine design. This approach was also utilised in HIV vaccines

303 design where removal of immunodominant CD8 T cell epitopes in a mosaic vaccine immunogen

serially up ranked subdominant epitopes which subsequently conferred efficacious T cell responses in mice challenge experiments (46).

Although HCV is recognised as one of the most genetically diverse human pathogens, significant regions of the viral genome are highly conserved across all known HCV subtypes. Presumably these conserved regions are highly constrained functionally during viral replication. Therefore, viral escape from T cells that target these regions is unlikely to develop without incurring a significant viral fitness cost, although viral escape is still possible particularly if the vaccine is not 100% efficacious. Furthermore, regions of high genomic conservation are likely to be also found in any future evolving HCV subtypes, such as the recently described genotype-7 and -8 and strains that are resistant to new directly anting antiviral therapies.

As conserved segment immunogens are chimeras which do not naturally occur, the junctions between conserved segments may potentially generate artificial non-natural T cell epitopes, with the potential to misdirect the T cell response away from relevant HCV T cell epitopes. Our previous *in silico* analysis demonstrated that the insertion of linker sequences would abrogate predicted strong binding of these artificial epitopes to their cognate TCR (33). We now show that the linker sequences displayed no immunogenicity *in vivo* as was predicted *in silico* and in fact their presence in the immunogen enhanced HCV-specific T cell response. The abrogation of strong-binding artificial epitopes through insertion of linker sequences may have altered with the immunopeptidome hierarchy of the vaccine infected cell allowing HCV epitopes to dominate naïve T cell induction.

A limitation of vaccines that utilise subdominant T cell epitopes may be the low frequency of naïve T cell populations for these epitopes or limitations in antigen presentation. Adjuvant strategies to enhance T cell responses to subdominant epitopes may be required to promote antigen presentation and greater expansion of naïve T cells. One of the most promising genetic vaccine adjuvants is the MHC class II invariant chain (Ii) which increases transgene-specific T cell responses when Ii is encoded directly upstream of the 5' end of the transgene (35,47,48). However, the use of non-human species specific Ii may be necessary to avoid autoimmunity in vaccinated humans, such as the truncated sequence of the shark invariant chain (sIi$_{tr}$; 24.6% sequence homology to hIi; 34). Here, we

334   demonstrated that inclusion of sli$_{tr}$ increased the magnitude and breadth of the vaccine-induced HCV-

335   specific T cell response. In other viral vector vaccine pre-clinical studies, sli$_{tr}$ enhanced the immune

336   response of viral vectors encoding malaria antigens (34). Whether sli$_{tr}$ increases the capacity of a T

337   cell vaccine to protect against HCV infection, remains to be shown.

338

339   In this study, we assessed a novel HCV vaccine strategy with the primary aim of inducing T cells to

340   conserved HCV sequences. The generation of HCV antibodies, following vaccination, was not

341   assessed since the immunogen and vaccine strategy was not designed to induce an antibody

342   response. Our aim rather, was to generate the most potent T cell vaccine possible, that may give

343   broad coverage against multiple HCV genotypes. We recognise that ultimately T cells alone may not

344   protect against HCV, and in the future vaccine strategies that aim to generate both T cell and

345   neutralising antibodies may need to be considered.

346

347   Furthermore, while our novel vaccine strategy induces T cell responses targeting conserved HCV

348   sequences that have also been identified in spontaneous resolvers, the evaluation of vaccine efficacy

349   is impeded by the lack of suitable small animal challenge models. While significant advances have

350   been made in humanised animal models that are permissible to HCV infection and suitable to assess

351   efficacious humoral immunity (49), an immunocompetent mouse model of chronic HCV infection, that

352   can support viral replication, to assess vaccine-induced protective T cell responses is not readily

353   available. Future efforts to develop a readily accessible, immunocompetent small animal model of

354   chronic HCV infection should be prioritised. Based on the data presented here, ChAd-Gt1-6L should

355   be the focus of future challenge studies and clinical trials in order to advance a single HCV vaccine for

356   global use through the clinical pipeline to be available to those who need it.

357

358   **MATERIALS AND METHODS**

359   **Vaccine nomenclature**

360   The ChAdOx1 conserved segment HCV T cell vaccines encode the conserved HCV sequence

361   segment of (1) genotype-1 and -3 subtypes and (2) all subtypes in genotype-1 to -6 as previously

362   described (33). They are referred to here as '*ChAd-Gt1/3*' and '*ChAd-Gt1-6*'. Both vaccines have long

363   and short immunogen versions, i.e. the shorter gt1/3 immunogen of 1000 amino acids is referred to as

13

364 '*ChAd-Gt1/3S*'. The longer gt1/3 immunogen of 1500 amino acids is referred to as '*ChAd-Gt1/3L*'. The

365 first-generation HCV T cell vaccine that encodes the genotype-1b non-structural sequence (NS3-5) is

366 referred to as ChAd-Gt1b-NS. A ChAd encoding the eGFP protein sequence was used as a vehicle

367 control. The conserved segment Gt1-6L vaccine without linkers between genomic segments is

368 referred to as ChAd-Gt1-6L_NL. Genetic adjuvants are described using suffixes on vaccine names,

369 for example, the shark invariant chain is ChAd-Gt1-6L-sIi.

370

371 **Animal experiments**

372 All mouse studies were performed at the Biomedical Services Building (BSB), Oxford, according to

373 UK Home Office Regulations (project license numbers 30/2744 and P874AC0FO) and approved by

374 the local ethical review board at the University of Oxford. All animal experiments complied with the

375 ARRIVE guidelines and were carried out in accordance with the UK Animals (Scientific Procedure)

376 Act, 1986. Groups of four to eight age-matched 6-8 week old female mice (BALB/c, C57BL/6, CD-1,

377 *HLA-A*02:01* transgenic mice) were used throughout and housed at a pathogen free facility in

378 individually-vented cages and fed a commercial block nutrient diet (Harlan Teklad Lab Blocks). Inbred

379 strains (the same H-2 haplotype, e.g. H-2K/D^b in C57BL/6 mice) were used to ensure limited immune

380 response variance between individual subjects in the same group. The outbred strain, CD-1, was

381 used to detect differences in the broad range of epitopes targeted by vaccine-induced T cell

382 responses (which may not be detected in inbred strains). *HLA-A*0201* transgenic mice were used to

383 assess the immunogenicity of conserved HCV epitopes when presented by human major

384 histocompatibility complex (MHC) receptors, to indicate if these vaccines may be immunogenic in

385 humans. After a 1-week adaptation period after arrival at the animal facility, mice were vaccinated

386 intramuscularly (IM, 26G needle) in the left quadricep with 40μL of viral vector vaccine solution (10^8

387 infectious units of vaccine in sterile PBS, immunised in the afternoon). Mice were harvested either 2-

388 or 3-weeks post vaccination by schedule 1 (CO_2 exposure followed by cervical dislocation).

389

390 **Peptides**

391 Peptides were obtained through BEI Resources, NIAID, NIH (genotype-1a H77, genotype-1b J4,

392 genotype-3a K3a650). These peptides were HCV genotype-specific 15-18mer synthetic peptides,

393 overlapping by 11 amino acids, and covering the length of the HCV proteome (optimal for CD4 and

394   CD8 T cell activation). Peptides were initially dissolved in dimethyl sulfoxide (DMSO) at 40mg/mL and

395   subsequently pooled into 10 pools at 300μg/mL labelled A (core), B (E1), C (E2), D (NS2), F (NS3p,

396   protease), G (NS3h, helicase), H (NS4), I (NS5a), L, (NS5bI, amino acids 2421-2718), and M (NS5bII,

397   amino acids 2719-3011). Peptide minipools (e.g. H1-H6), segment pools (S1-S25 matching

398   conserved Gt1-6L sequence segments), and individual peptides containing *HLA-A*02:01* epitopes

399   (described in human studies and reported in the Los Alamos database) were generated to stimulate

400   splenocytes in ELISpot assays.

401

402   **Splenocyte isolation**

403   Harvested mouse splenocytes were harvested immediately after schedule 1 killing (CO_2 exposure

404   followed by cervical dislocation) and collected in ice cold PBS. Lymphocytes were isolated by

405   mechanical processing using a sterile plunger and 40μm cell strainer. Red blood cells were lysed with

406   ACK lysis buffer for no longer than one minute and remaining cells resuspended in R10 media (RPMI

407   1640 media with L-glutamine (5%), penicillin-streptomycin (5%), and 10% foetal calf serum). Cell

408   yields were calculated using a Guava Personal Cell analysis system (Merck Millipore 0100-14230)

409   and the Muse® Cell Analyser (Merck Millipore). The machine was calibrated prior to cell counting

410   using Guava check beads (16-0040).

411

412   *Ex vivo* **IFNγ ELISpots**

413   Multiscreen®$_{HTS}$ IP filter plates (PVDF; Merk Millipore) were pre-wetted with 20μL of 35% ethanol per

414   well for no longer than 60 seconds. Plates were washed with PBS and pre-coated with anti-mouse

415   anti-IFNγ mAb (AN18, 0.5μg/well, 1:200 dilution, Mabtech, Sweden) overnight, then washed and

416   blocked with R10 for two hours at 37°C. After blocking, cells were plated at 1-2x10^5 cells per well in

417   50μL R10 media and stimulated for 20-24 hours at 37°C with 50μL HCV genotype-1a, -1b, -3a

418   peptide pools, minipools, or peptides (3μg/mL final peptide concentration in 100μL total R10 media;

419   NIH, MD, USA), a DMSO negative control without HCV peptides to measure background IFNγ^+ SFU

420   responses and a concanavalin positive control (conA, 10μg, Sigma). Bound IFNγ was detected using

421   anti-mouse IFNγ mAb R4-6A2 biotinylated (1:2000 dilution, Mabtech, Sweden), anti-biotin alkaline

422   phosphatase (1:750, Vector Laboratories, Burlingame, CA, USA), and BCIP/NBT phosphatase

423   substrate (Thermo Scientific, IL, USA). T cell responses are reported as IFNγ^+ SFU/10^6 splenocytes and

424   the total T cell magnitude is the sum SFU of the positive individual peptide pools minus the mean DMSO

425   SFU multiplied by the number of positive peptide pools. Peptide pools are considered positive when

426   greater than the mean of the DMSO negative control plus three standard deviations. Antibody details can

427   be found in the **supplementary table 2**.

428

429   **Intracellular cytokine staining**

430   Splenocytes were stimulated using HCV genotype-1a, -1b, and -3a peptide pool combinations

431   (A+B+C = Core, E1, E2; F+G+M = NS3-4, I+L+M = NS5a-b, 1.5μg/mL, 15-18mers overlapping by 11

432   amino acids). An negative control (DMSO) and PMA (phorbol 12-myristate 13-acetate)/ionomycin

433   positive control (50 and 500ng/mL, respectively) were used. Cells were stimulated for 6 hours with

434   peptide pools (4μg/mL GolgiPlug^{TM} (BD Biosciences) was added for the last 4 hours of the

435   stimulation). Cells were stained with fixable Near-IR live/dead dye (Life Technologies, USA), CD3-

436   efluor450, CD4-AlexaFluor700, CD8-peridinin chlorophyll protein (PerCP) Cy5.5 for 30 minutes at

437   4°C, before being fixed and permeabilised with fixation/permeabilization solution (BD Biosciences) at

438   4°C for 10 minutes. Following fixation, cells were stained with IFNγ-phycoerythrin (PE), TNFα-

439   fluorescein isothiocyanate (FITC), and IL-2-oallophycocyanin (APC) at 4°C for 30 minutes, and

440   subsequently washed and run on the LSRII flow cytometer. ICS data was corrected for background by

441   subtracting the cytokine production as a percentage of CD4$^+$ or CD8$^+$ T cell subsets in a matched

442   DMSO negative control. Gating and analysis were performed in FlowJo (TreeStar, v10.5, USA).

443   FlowJo Boolean gating was used for cytokine co-expression and graphs produced in Pestle (v1.8),

444   and SPICE (NIAID, NIH, v5.35). Antibody details can be found in the **supplementary table 2**.

445

446   **Sequence analysis**

447   HCV amino acid sequences were aligned and analysed in Aliview (version 1.18). HCV subtype amino

448   acid sequences (International Committee on the Taxonomy of Viruses, May 2019) were obtained from

449   UniProt.org. Basic Local Alignment Search Tool (BLAST) analysis was done using the protein BLAST

450   tool (NCBI; National Centre for Biotechnology Information website).

451

452   **Statistical analysis**

453   Data were analysed using GraphPad prism (version 8.0.1). Preliminary studies were undertaken to

454   determine appropriate sample sizes. The D'Agostino and Pearson test was used to determine data

455   distribution normality. Unless otherwise stated, non-parametric tests (Mann Whitney or Kruskal-Wallis

456   test) were used to determine significant difference between two group medians at 95% confidence

457   intervals between two or more groups, respectively. P values less than 0.05 indicate a significant

458   difference: $p < 0.05$ = *, <0.01 = **, <0.001 = ***, and <0.0001 = ****. Only statistical differences

459   (asterisks) are displayed.

460

461   **AUTHOR CONTRIBUTIONS**

462   **Timothy Donnison**: Methodology, Conceptualisation, Investigation, Formal analysis, Visualisation,

463   Project administration, Writing – Original Draft. **Anette von Delft**: Conceptualisation, Writing – Review

464   & Editing. **Anthony Brown**: Methodology, Resources, Investigation. **Leo Swadling**: Investigation,

465   Writing – Review & Editing. **Claire Hutchings**: Methodology, Resources, Investigation, Writing –

466   Review & Editing. **Tomáš Hanké**: Concenptualisation, Writing – Review & Editing. **Senthil**

467   **Chinnakannan**: Supervision, Resources, Investigation, Writing – Review & Editing. **Eleanor Barnes**:

468   Funding acquisition, Project administration, Supervision, Writing – Review & Editing.

469

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474

475   **FUNDING SOURCES**

482

483 **CONFLICTS OF INTEREST**

484 TD, AvD, SC, and EB are all contributors or inventors on patents for the conserved segment HCV T

485 cell vaccines.

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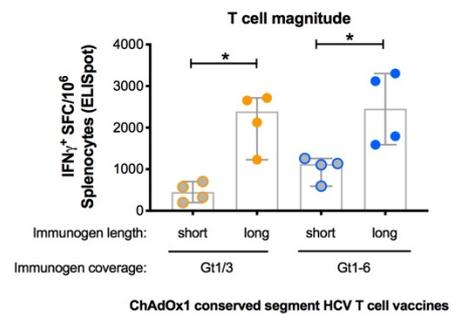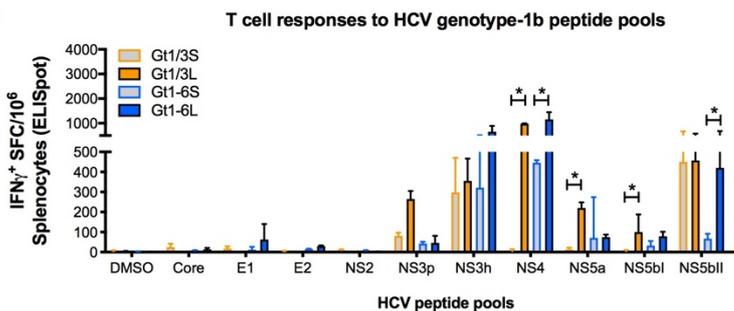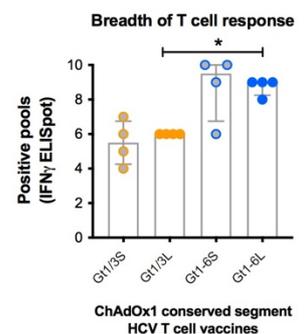
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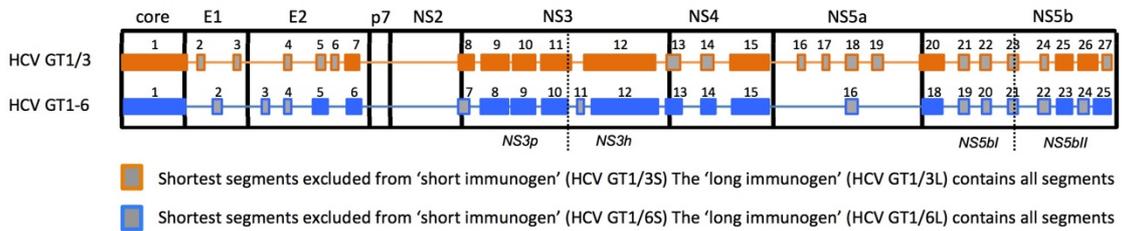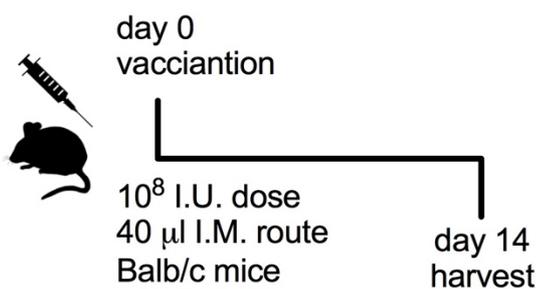
FIGURES


Figure 1. Immunogen design, in vitro expression and in vivo vaccine immunogenicity of

conserved segment HCV T cell vaccines: (**A**) Conserved segment HCV T cell immunogens that

contain gene segments that correspond to conserved viral sequences across viral genotypes 1 and 3,

and 1 to 6. Segments are numbered left to right. Light grey segments surrounded by a dark grey

border correspond to shorter length gene segments that are excluded from the short immunogens but

are included in the long immunogens. (**B**) Conserved segment HCV T cell vaccines were evaluated for

immunogenicity in 8-week old female *BALB/c* inbred mice (4/group) that were immunised with a single 10^8

infectious units (IU) intramuscular immunisation in the left quadricep and measured two weeks post-

vaccination. (**C**) The total T cell magnitude to all HCV peptide pools as determined by IFNγ-producing

639 SFU/10^6 splenocytes in an *ex vivo* ELISpot assay. (**D-E**) The breadth of the T cell response to all HCV

640 peptide pools. For ELISpot assays, harvested splenocytes were stimulated with HCV genotype-1b (J4)

641 peptide pools (final concentration of 3μg/ml) that cover the full length of the HCV proteomic sequence (15-

642 18mers overlapping by 11aa). Data presented includes 'short' or 'long' versions of the vaccines 'gt1/3' and

643 'gt1-6'. Bars represent the median SFU/10^6 splenocytes, with interquartile ranges displayed. *P* values

644 (Mann Whitney tests) indicate significant difference between two groups when < 0.05*. Only statistically

645 significant differences between groups, indicated by an asterisk, are shown.
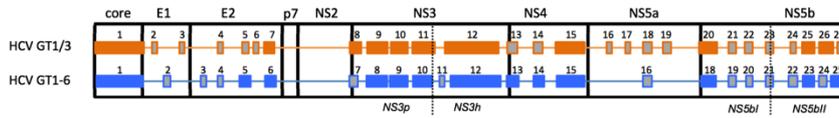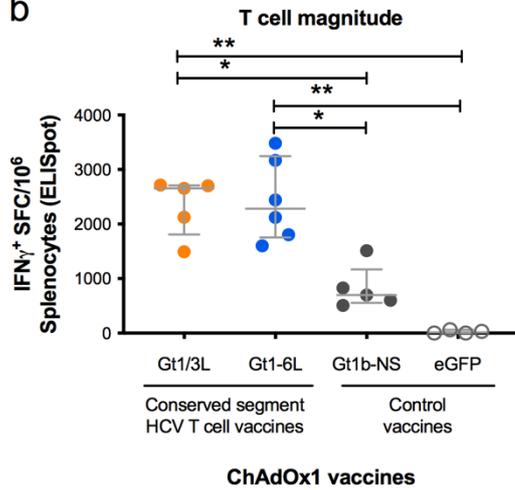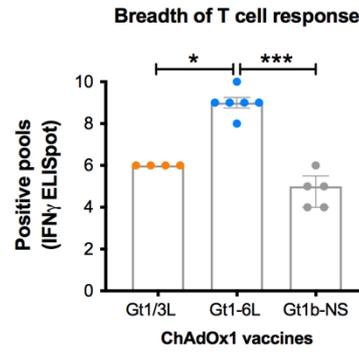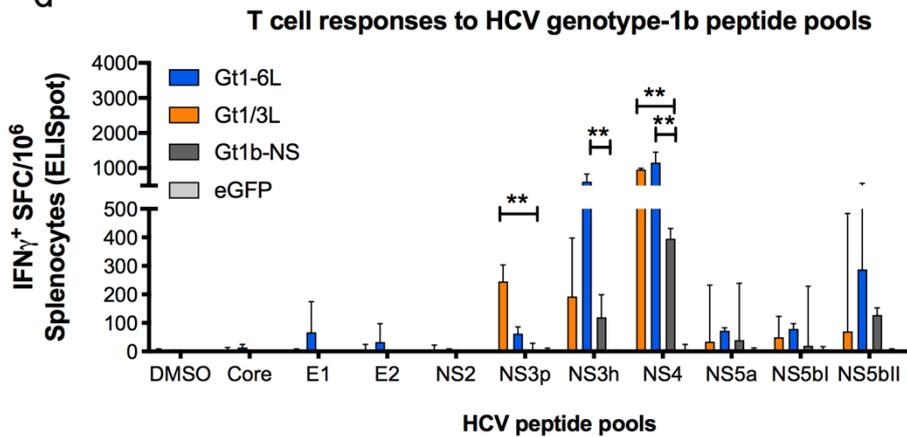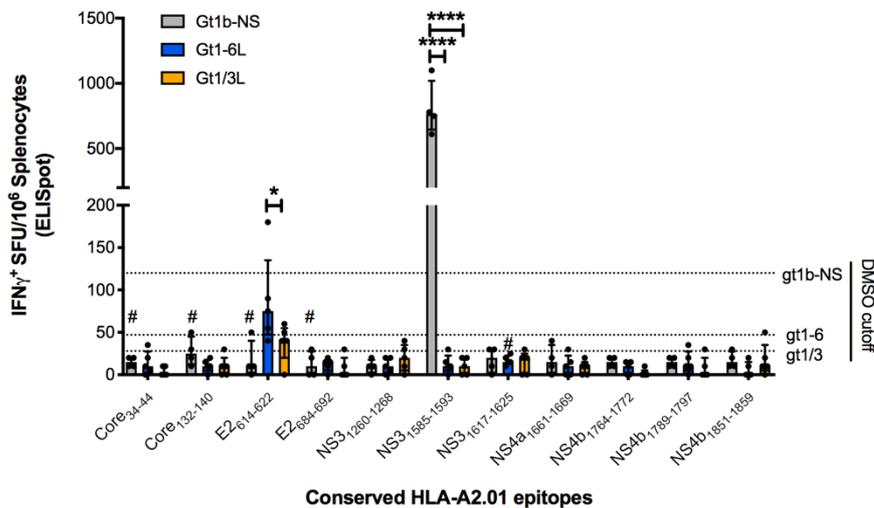
647 **Figure 2. Comparative assessment of vaccine-induced T cell responses between conserved**

648 **segment HCV T cell vaccines and subtype-1b HCV T cell vaccine**: HCV T cell vaccine

649 immunogen design (**A**). The ex vivo IFNγ ELISpot assay response for vaccine-induced T cell

650 magnitude (**B**), breadth by number of positive peptide pools (**C**), and T cell magnitude to individual

651 peptide pools across the full length of the HCV proteome (15-18mers overlapping by 11aa; **D**). Four to

652 six female age matched *BALB/c* mice were vaccinated per group, each mouse receiving 10^8 IU of

653 vaccine in a 40μL intramuscular injection and harvested 14 days post-vaccination. Bars represent the

654 median SFU/10^6 splenocytes, with interquartile range displayed. Data is combination of two

655 experiments. (**E**) The IFNg ELISpot response to previously identified HLA-A*0201 restricted HCV

656 epitopes in *HLA-A*02:01* transgenic mice (5 mice/group received 10^8 IU single intramuscular

657 immunisation and were harvested 14 days post-vaccination) stimulated with genotype-1b peptides

658 (15-18mer) containing *HLA-A*02:01* identified epitopes described in human studies and reported in

659 the Los Alamos database. Hashes indicate epitopes that are not present in the vaccine immunogen. The

660 experiment was performed once. Pools in all experiments are defined as positive when greater than the

661 mean of the DMSO negative control plus three standard deviations. Bars represent the median SFU/10^6

662 splenocytes, with interquartile range displayed. Kruskal-Wallis tests with multiple comparisons were

663 performed to determine a significant difference between two group medians at a 95% confidence interval.

664 *P* values indicate significant difference between groups when < 0.05*, <0.01**, <0.001***, <0.0001****.

665 Only statistically significant differences between groups, indicated by an asterisk, are shown.
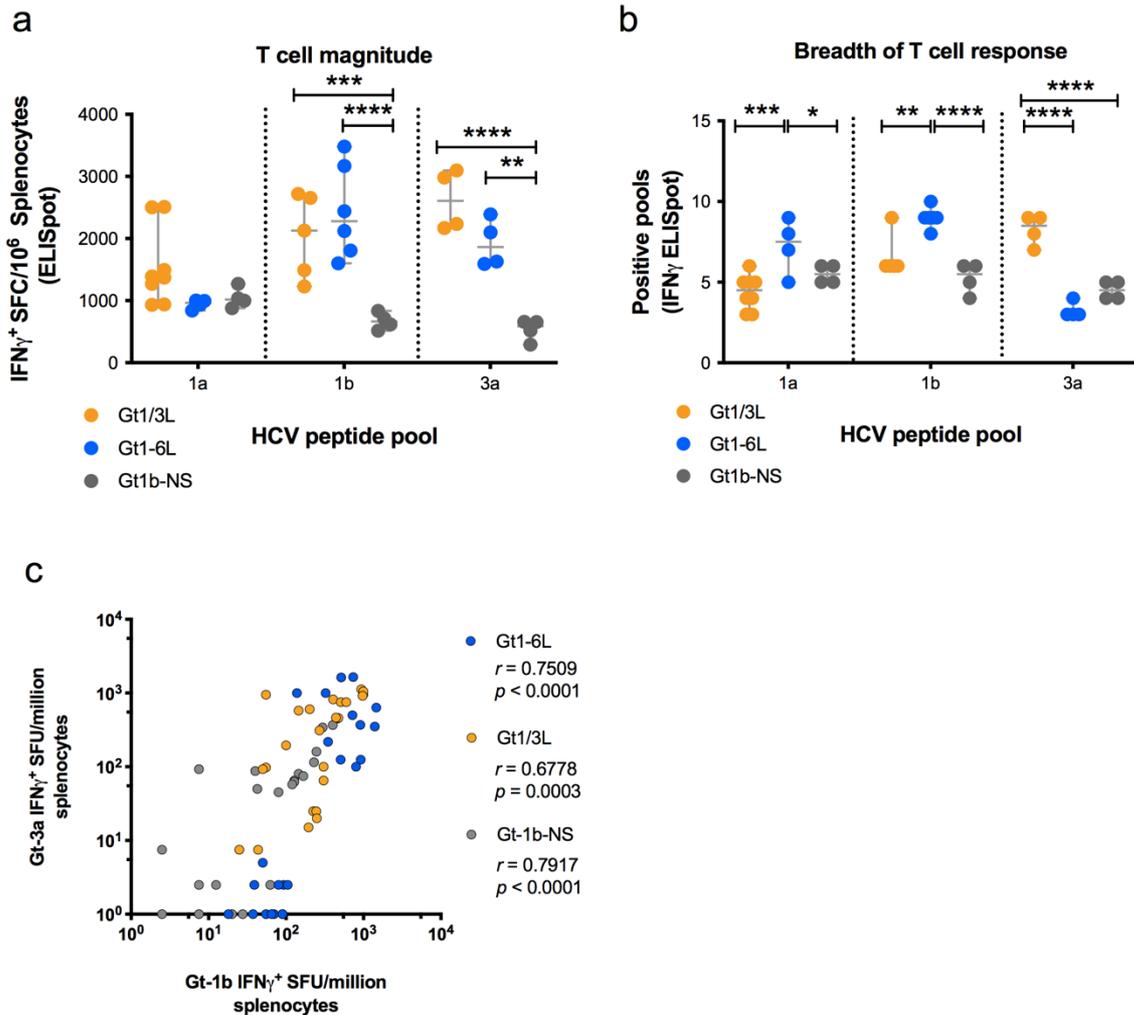
Figure 3. Intergenotypic T cell responses induced by HCV T cell vaccines: Splenocytes from age-matched female *BALB/c* mice (n=4-8/group) that received a single 10^8 IU vaccine dose in a 40µL intramuscular immunisation with three different HCV T cell ChAd vaccines (gt-1b-NS, gt1-6L, gt1/3) were harvested 14 days post-vaccination and stimulated with genotype-1a (H77), -1b (J4), and -3a (k3a650) peptides in 10 pools and IFNγ producing cells were detected by *ex vivo* IFNγ ELISpot for comparison of T cell magnitude (**A**) and number of positive peptide pools (**B**) and correlation between genotype-1b and -3a T cell responses (**C**). Bars represent the median SFU/10^6 splenocytes, with interquartile range displayed. The data is a combination of two experiments. A two-way ANOVA with multiple comparisons was used to determine statistical significance between groups at a 95% confidence interval. *P* values indicate significant difference between groups when < 0.05*. Only statistically significant differences between groups, indicated by an asterisk, are shown.

678
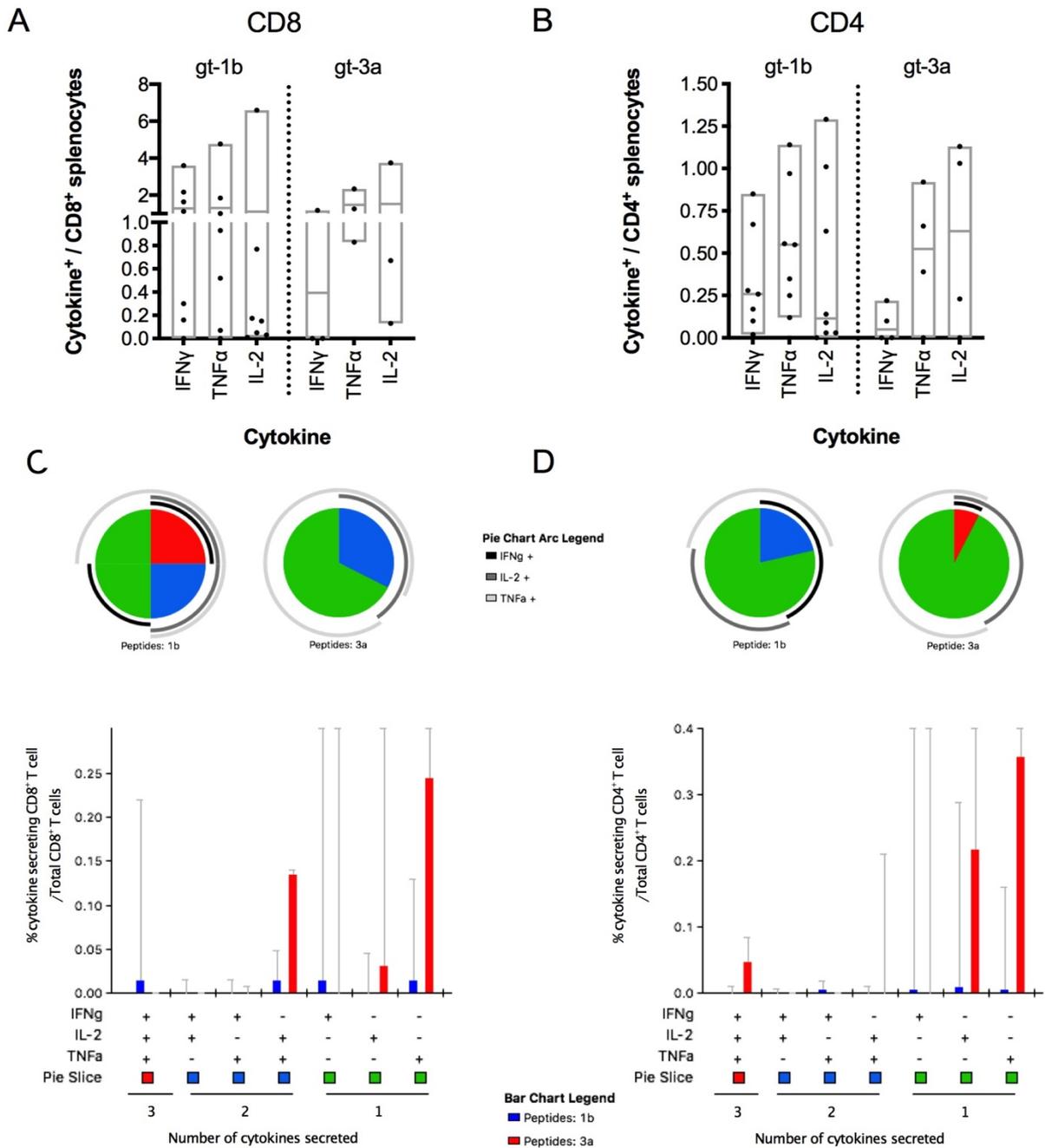
Figure 4. Functionality of vaccine-induced HCV genotype-1b and -3a specific T cell responses:
Intracellular staining of IFNγ, TNFα, and IL-2 produced by splenic CD4⁺ and CD8⁺ T cells from age-matched female *C57BL/6* mice immunised with 10^8 IU IM prime ChAd-gt1-6L vaccination and harvested 14 days post-vaccination (n=3-8; data presented are from two experiments). Cells were stimulated with either genotype-1b or -3a specific HCV peptides in 3 pools that cover the full HCV protein sequence (i. HCV core-E1-E2, ii. NS3-4, and iii. NS5; peptides are 15-18mers overlapping by 11aa). Cytokine production of vaccine induced CD8⁺ (**A**) and CD4⁺ (**B**) T cells is shown and displayed

686   as the sum response of all three peptide pools in the left column as floating box plots with medians

687   shown). Cytokine secreting CD8$^+$ (**C**) and CD4$^+$ (**D**) T cell subsets were analysed for polyfunctionality

688   using Boolean gating, Pestle software and SPICE analysis. Pie charts and graphs represent the

689   proportion of cytokine-secreting T cells that produce one (light grey), two (dark grey), or three (black)

690   cytokines of IFNγ, TNFα, and IL-2. Pie arcs (in greyscale) show the proportion of cytokine-producing

691   cells that make a given cytokine, where overlap of arcs indicate polyfunctionality. Pie bases and bars

692   are displayed as medians of all samples, with interquartile ranges displayed.
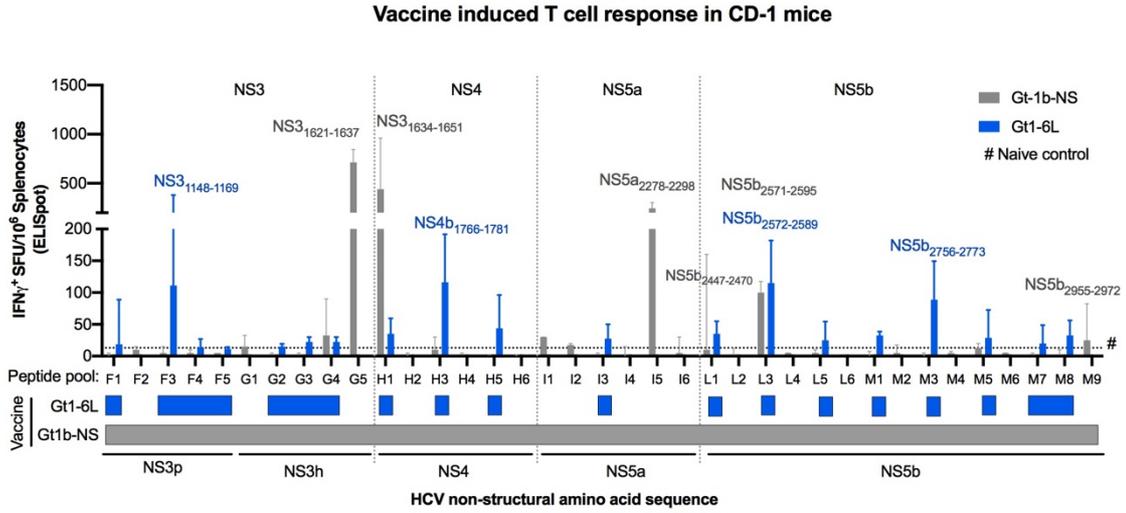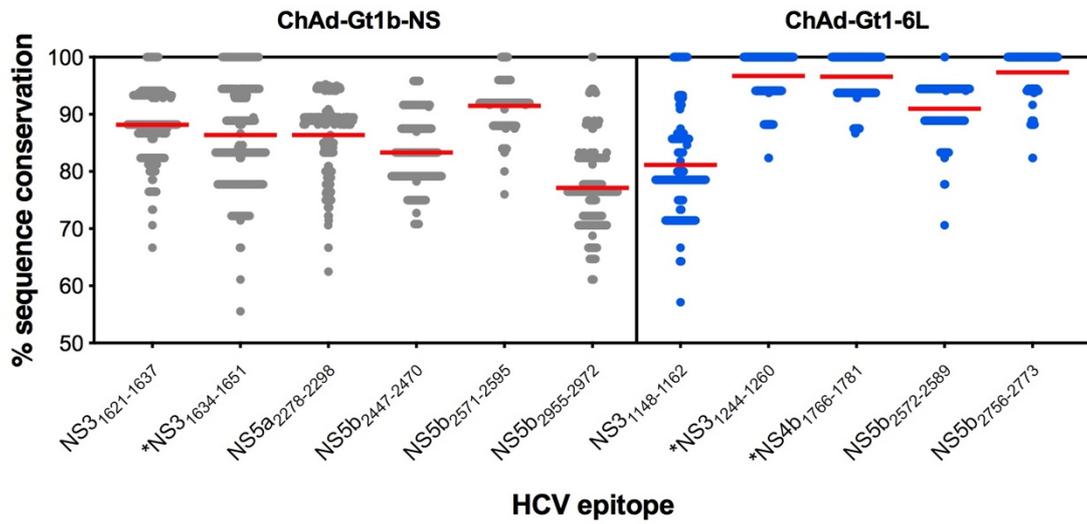
Figure 5. Comparative analysis of ChAdOx1-gt-1b-NS and ChAdOx1-gt1-6 vaccine-induced immunogenicity to conserved HCV epitopes: Age-matched female *CD-1* mice were vaccinated

696   with 10^8 IU of either ChAd-Gt1b-NS or ChAd-gt1-6L in a 40uL intramuscular injection and harvested

697   3-weeks post vaccination (n=4-8 for each vaccine, respectively, and the experiment was performed

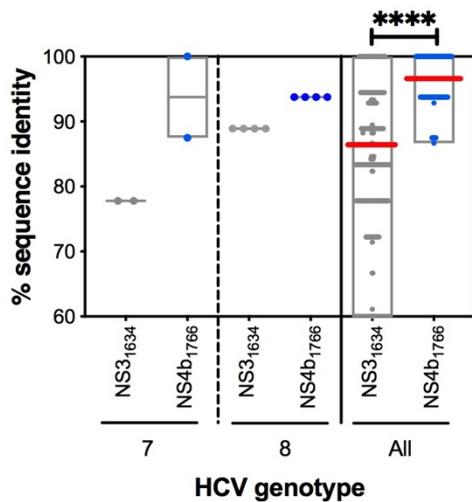698   twice). (**A**) The breadth of the vaccine-induced T cell response to peptide minipools that cover the

699   subtype-1b NS proteome (15-18mers overlapping by 11aa). (**B**) The percentage sequence

700   conservation (number of amino acids that are difference as a percentage) of vaccine-induced T cell

701   targeted epitopes across HCV subtypes with means displayed (listed and defined by the International

702   Committee for the Taxonomy of Viruses [ICTV] as of May 2019). The asterisk (*) indicates epitopes

703   that were identified in *C57BL/6* mice in a separate experiment. (**C**) The percentage sequence

704   conservation of $NS3_{1634}$ and $NS4_{1766}$ epitope sequences across HCV genotype-7 and -8 with means

705   and ranges displayed. *P* values (Mann Whitney tests) indicate significant difference between groups

706   when < 0.05*, <0.01**, <0.001***, <0.0001****. Only statistically significant differences between groups,

707   indicated by an asterisk, are shown.

a

Invariant chain transmembrane domain sequence

Human Homology:

human: gal-ytgfsilvtlllagqattayfly   100% hIi

shark: sll-wggvtvlaamliagqvasvvflv   24.6% sli

sli$_{tr}$

core | E1 | E2 | p7 | NS2 | NS3 | NS4 | NS5a | NS5b

Gt1-6L immunogen

b

T cell magnitude

SFU/10^6 IFNγ$^+$ Splenocytes (ELISpot)

ChAdOx1-Gt1-6L adjuvented vaccines

TPA-LS   sli$_{tr}$ No GA

*

c

T cell breadth

No. of positive peptide pools (ELISpot)

ChAdOx1-Gt1-6L vaccine variants

TPA-LS   sli$_{tr}$ No GA

**

d

T cell responses to gt1-6 conserved segments (S1-25)

SFU/10^6 IFNγ$^+$ Splenocytes (ELISpot)

TPA-LS
sli$_{tr}$
No GA

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

C | E1 | E2 | NS3 | NS4 | NS5

*
*
*

Segment peptide pools (gt1-6L)

e

CD-1

IFNγ$^+$ SFC/10^6 Splenocytes (ELISpot)

1a   1b   3a

*

*

HCV genotype stimuli

Gt1-6L-TPA-LS
Gt1-6L-sli$_{tr}$

708

33

Figure 6. The design of HCV viral vector vaccines with genetic adjuvant truncated shark invariant chain (sli$_{tr}$) and vaccine immunogenicity in *C57BL/6* and *CD-1* mice: (**A**) A schematic of the truncated shark invariant chain (sli$_{tr}$) sequence and truncated human invariant chain sequence alignment (hli$_{tr}$). Sli$_{tr}$ is encoded at the 5' end of the gt1-6L HCV conserved immunogen sequence. *C57BL/6* or *CD-1* mice (4-6/group) were vaccinated with 10^8 IU of ChAd-Gt1-6L-TPA-LS ('*TPA-LS*', tissue plasminogen activation leader sequence), ChAd-Gt1-6L-sli ('*sli$_{tr}$*'), or ChAd-Gt1-6L (no genetic adjuvant, '*No GA*') in a 40μL intramuscular injection and harvested 2-weeks post vaccination. (**B**) T cell magnitude and (**C**) the number of IFNγ positive ELISpot pools in *C57BL/6* mice. (**D**) The breadth of the vaccine-induced T cell response to conserved gt1-6 sequence peptide pools (S1-25) that cover the subtype-1b specific conserved sequence segments of the Gt1-6L immunogen in *C57BL/6* mice (15-18mers overlapping by 11aa). (**E**) Genotype-1a, -1b, and -3a specific T cell responses of murine splenocytes isolated from *CD-1* outbred mice 3-weeks post-vaccination with 10^7 IU IM ChAdOx1-Gt1-6L vaccines. Bars represent the median SFU/10^6 splenocytes, with interquartile range displayed. Kruskal-Wallis tests with multiple comparisons were performed to determine a significant difference between two group medians at a 95% confidence interval. *P* values indicate significant difference between groups when < 0.05*, <0.01**, <0.0005***, <0.0001****. Only statistically significant differences between groups, indicated by an asterisk, are shown.

726 TABLES

HCV vaccine	Mouse strain	Peptide pool (segment/minipool)	Immunogenic HCV peptide (gt-1b)	Peptide location (H77 ref)	Peptide % sequence conservation across all HCV subtypes	Identified in human SRs * (HCV subtype)
Gt1-6L	CD-1, HLA-A2	C (Seg5)	RCMVDYPYRLWHYPCTI	E2 606-622	86.7	Yes (1/3a)
	CD-1	F (Seg8/F3)	SRGSLLSPRPISYLK	NS3 1148-1162	81.2	Yes
	C57BL6	F (Seg9/F4)	YAAQGYKVLVLNPSVAA	NS3 1244-1260	96.7	Yes (1a/1b/3/4)
	C57BL6, CD-1	H (Seg14/H3)	WNFISGIQYLAGLSTL	NS4b 1766-1781	96.6	Yes (1a/1b/3/4)
	CD-1	L (seg19/L3)	GGRKPARLIVYPDLGVRV	NS5b 2572-2589	91.0	Yes (1a/1b/3)
	CD-1	M (Seg22/M3)	LRAFTEAMTRYSAPPGDP	NS5b 2756-2773	97.3	No
Gt1b-NS	HLA-A2	G (Seg12/G4)	FPYLVAYQATVCARAQA	NS3 1583-1599	94.2	Yes
	CD-1	G (G5)	PTPLLYRLGAVQNEVIL	NS3 1621-1637	88.2	Yes (1/1b/3)
	C57BL6, CD-1	H (H1)	EVTLTHPITKYIMACMSA	NS3 1634-1651	86.4	No
	CD-1	I (I5)	SRKFPSALPIWARPDYNPPLL	NS5a 2278-2298	86.4	Yes (1/1a)
	CD-1	L (L1)	SNSLLRHHNMVYATTSRSASLRQK	NS5b 2447-2470	83.3	Yes (1/3)
	CD-1	L (L3)	KGGRKPARLIVFPDLGVRVCEKMAL	NS5b 2571-2595	91.5	Yes (1/1b/3)
	CD-1	M (M9)	KLTPIPAASQLDLSGWFV	NS5b 2955-2972	77.1	Yes (1a/1b)

727

728 **Table 1. HCV epitopes targeted by vaccine-induced T cells**: HCV sequences targeted by vaccine-induced T cells are displayed with corresponding peptide

729 pool, mouse strain detected in, percentage sequence conservation across all HCV subtypes (ICTV May 2019 database), and epitopes, if any, have been

730 identified in HCV spontaneous resolution in humans. * Epitopes were detected by 90% sequence blast search on IEDB.org and only included if they contain

731 at least eight overlapping amino acids with their respective peptide sequence. Note, no evidence of epitopes reported for gt-2,4, and 5 was identified, likely

732 reflecting the lack of cohorts for which spontaneous resolution of these genotypes has been reported.

733 **SUPPLEMENTARY FIGURES AND TABLES**

734 Supplementary figure 1. The effect of linker sequences between gt1-6 gene segments on vaccine-

735 induced immunogenicity

736 Supplementary figure 2. Flow cytometry plots of intracellularly stained murine splenocytes

737 Supplementary figure 3. HCV vaccine peptide-specific T cell responses in *CD-1* outbred mice

738 Supplementary figure 4. Comparative analysis of T cell HCV epitopes across genotype-1 to -6

739 Supplementary figure 5. Comparative analysis of ChAd-Gt1b-NS and ChAd-Gt1-6 vaccine-induced

740 immunogenicity to conserved HCV sequences

741 Supplementary table 1. *HLA-A*02:01*-restricted HCV-derived epitope sequences

742 Supplementary table 2. Antibodies, software, repositories, and vectors

743

a

HCV GT1-6

core   E1   E2   p7   NS2   NS3   NS4   NS5a   NS5b

L1 L2 L3 L4 L5 L6   L7 L8 L9   L10 L11   L12 L13 L14   L15 L16 L17

■ Shortest segments excluded from 'short immunogen' (HCV GT1-6S).
The 'long immunogen' (HCV GT1-6L) contains all segments (1-25)

↑ Linker sequences of glycine, lysine, proline and serine.
The 'long immunogen' (HCV GT1-6L_NL) contains all segments (1-25) and no linker sequences

b

T cell magnitude

c

Breadth of T cell response

d

T cell responses to HCV genotype-1b peptide pools

e

Linker-specific T cell response

746 **Supplementary figure 1. The effect of linker sequences between gt1-6 gene segments on**

747 **vaccine-induced immunogenicity**: The conserved segment vaccine, ChAd-gt1-6, with linker

748 sequences. Red arrows indicate the location of all 17 linkers (L1-17) that were inserted between

749 conserved gt1-6 gene segments (**A**). The *ex vivo* IFNg ELISpot assay for vaccine-induced T cell

750 magnitude (**B**), breadth indicated by number of positive peptide pools (**C**), T cell magnitude of

751 individual peptide pools (**D**), and vaccine-induced immunogenicity to linker sequences in junction

752 regions between HCV gene segments (**E**). DMSO, medium (R10), and Concanavalin A (ConA) were
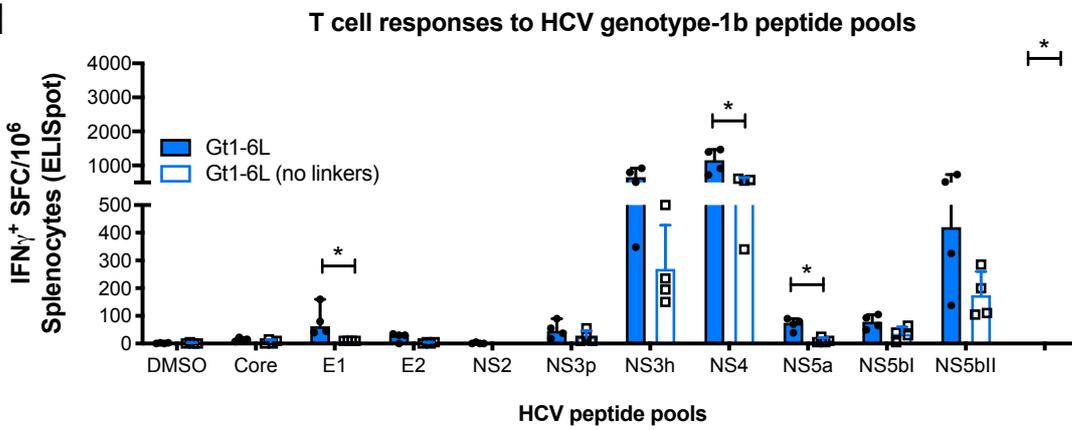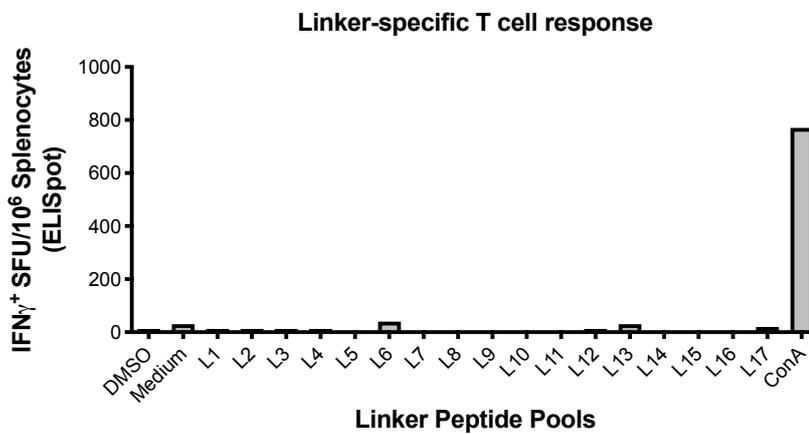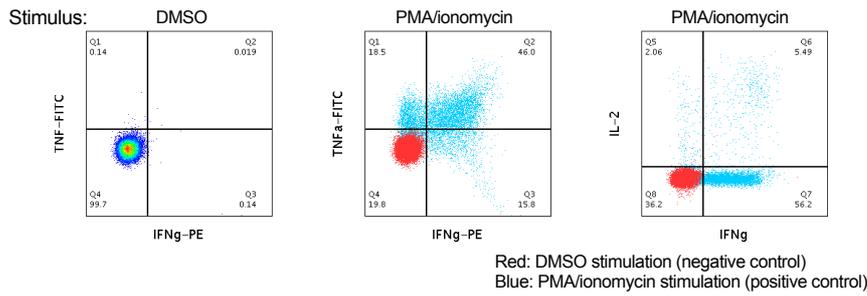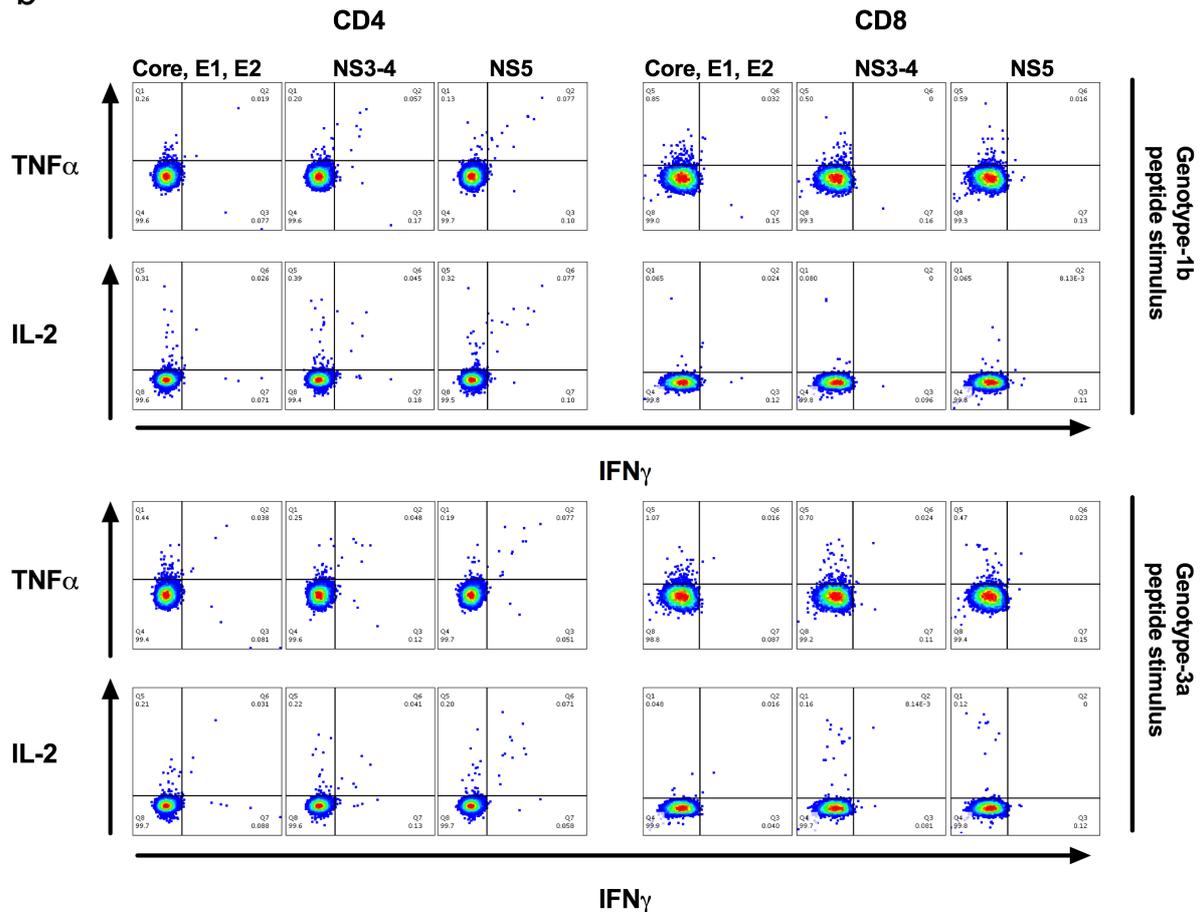
753 used as two negative controls and a positive control, respectively. T cell magnitude is the total of the

754 positive individual peptide pools. Pools are considered positive when greater than the mean of the DMSO

755 negative control plus three standard deviations. Four female age-matched *BALB/c* mice were vaccinated

756 per group, each mouse receiving 10^8 IU of ChAd-gt1-6L in a 40μL intramuscular injection and harvested

757 14 days post-vaccination. Bars represent the median SFU/10^6 splenocytes, with interquartile range

758 displayed. Mann Whitney tests were performed to determine a significant difference between two group

759 medians at a 95% confidence interval. *P* values indicate significant difference between groups when <

760 0.05*, <0.01**, <0.001***, <0.0001****.

761

a

Stimulus: DMSO / PMA/ionomycin / PMA/ionomycin

Red: DMSO stimulation (negative control)
Blue: PMA/ionomycin stimulation (positive control)

b

CD4 — Core, E1, E2 / NS3-4 / NS5
CD8 — Core, E1, E2 / NS3-4 / NS5

TNFα / IL-2

Genotype-1b peptide stimulus

IFNγ

TNFα / IL-2

Genotype-3a peptide stimulus

IFNγ

762

Supplementary figure 2. Flow cytometry plots of intracellularly stained murine splenocytes: (**A**) Gating strategy to identity CD4+ and CD8+ T cell subset cytokine production using DMSO (negative) and PMA/ionomycin (positive) controls. (**B**) Example plots of intracellular staining of IFNg, TNFa, and IL-2 produced by splenic CD4+ and CD8+ T cells from age-matched female *C57BL/6* mice immunised with 10^8 IU I.M. prime ChAd-gt1-6L vaccination and harvested 14 days post-vaccination. Cells were stimulated with either genotype-1b or 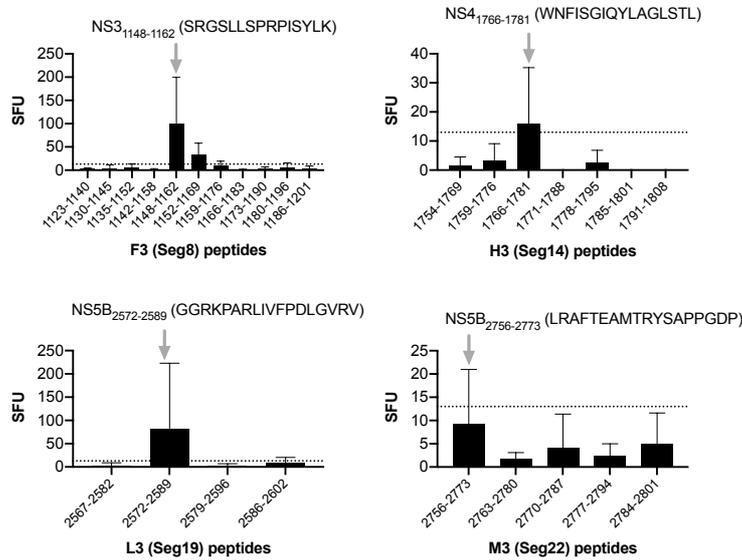-3a specific HCV peptides in 3 pools that cover the full HCV protein sequence (i. HCV core-E1-E2, ii. NS3-4, and iii. NS5; peptides are 15-18mers overlapping by 11aa).

771

772

a **ChAd-Gt1-6L in CD-1 mice**

NS3$_{1148-1162}$ (SRGSLLSPRPISYLK)

NS4$_{1766-1781}$ (WNFISGIQYLAGLSTL)

F3 (Seg8) peptides

H3 (Seg14) peptides

NS5B$_{2572-2589}$ (GGRKPARLIVFPDLGVRV)

NS5B$_{2756-2773}$ (LRAFTEAMTRYSAPPGDP)

L3 (Seg19) peptides

M3 (Seg22) peptides

b **ChAd-Gt1b-NS in CD-1 mice**

NS3$_{1621-1637}$ (PTPLLYRLGAVQNEVIL)
NS3$_{1634-1651}$ (EVILTHPITKYIMACI)

NS5A$_{2278-2293}$ (SRKFPSALPIWARPDY)
NS5A$_{2283-2298}$ (SALPIWARPDYNPPL)

NS5B$_{2447-2463}$ (SNSLLRHHNMVYATTSR)
NS5B$_{2453-2470}$ (HHNMVYATTSRSASLRQK)

Peptides

I5 peptides

L1 (NS5b) peptides

NS5B$_{2571-2588}$ (KGGRKPARLIVFPDLGVR)
NS5B$_{2578-2595}$ (RLIVFPDLGVRVCEKMAL)

NS5B$_{2955-2972}$ (KLTPIPAASQLDLSGWFV)

L3 peptides

M9 peptides

773

774 **Supplementary figure 3**. **HCV vaccine peptide-specific T cell responses in *CD-1* outbred mice**:

775 Groups of 4 age-matched female *CD-1* mice were vaccinated with 10^8 IU of vaccine in a 40uL

776 intramuscular injection and harvested 5-weeks post vaccination. The experiment was performed

777 once. Vaccine immunogenicity of ChAd-Gt1-6L (**A**) and ChAd-Gt1b-NS (**B**) in *CD-1* outbred mice

778 splenocytes stimulated with individual peptides of positive peptide minipools. Pools are considered

779   positive when greater than the mean of the DMSO negative control plus three standard deviations and
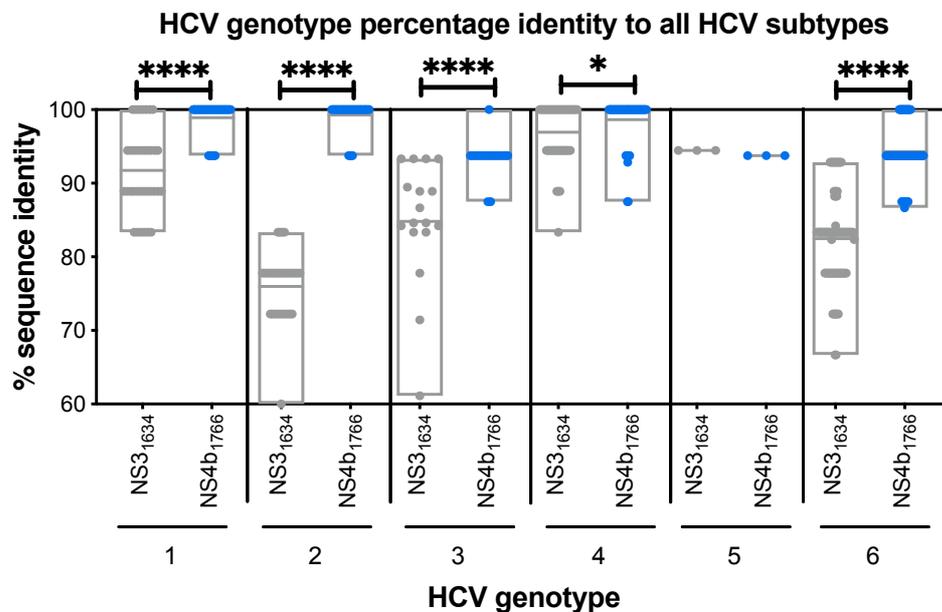
780   greater than the *CD-1* naïve unvaccinated control ELISpot response. Peptides are 15-18mers, overlap

781   by 11aa, and cover the genotype-1b specific NS proteome. Bars represent the median SFU/10^6

782   splenocytes, with interquartile range displayed.

HCV genotype percentage identity to all HCV subtypes

783

784   **Supplementary figure 4. Comparative analysis of T cell HCV epitopes across genotype-1 to -6:**

785   The percentage sequence identity when comparing $NS3_{1634}$ and $NS4_{1766}$ epitope sequences across

786   HCV genotypes 1 to 6 with the epitope sequence in each vaccine (each HCV subtype is a data point)

787   with means and ranges displayed (subtypes listed and defined by the International Committee for the

788   Taxonomy of Viruses [ICTV] as of May 2019). The $NS3_{1634}$ epitope was targeted by the ChAd-Gt1b-NS

789   vaccine whereas the $NS4_{1766}$ epitope was targeted by the ChAd-Gt1-6L vaccine. Mann Whitney tests were

790   performed to determine a significant difference between two group medians at a 95% confidence interval.

791   *P* values indicate significant difference between groups when < 0.05*, <0.01**, <0.001***, <0.0001****.

792
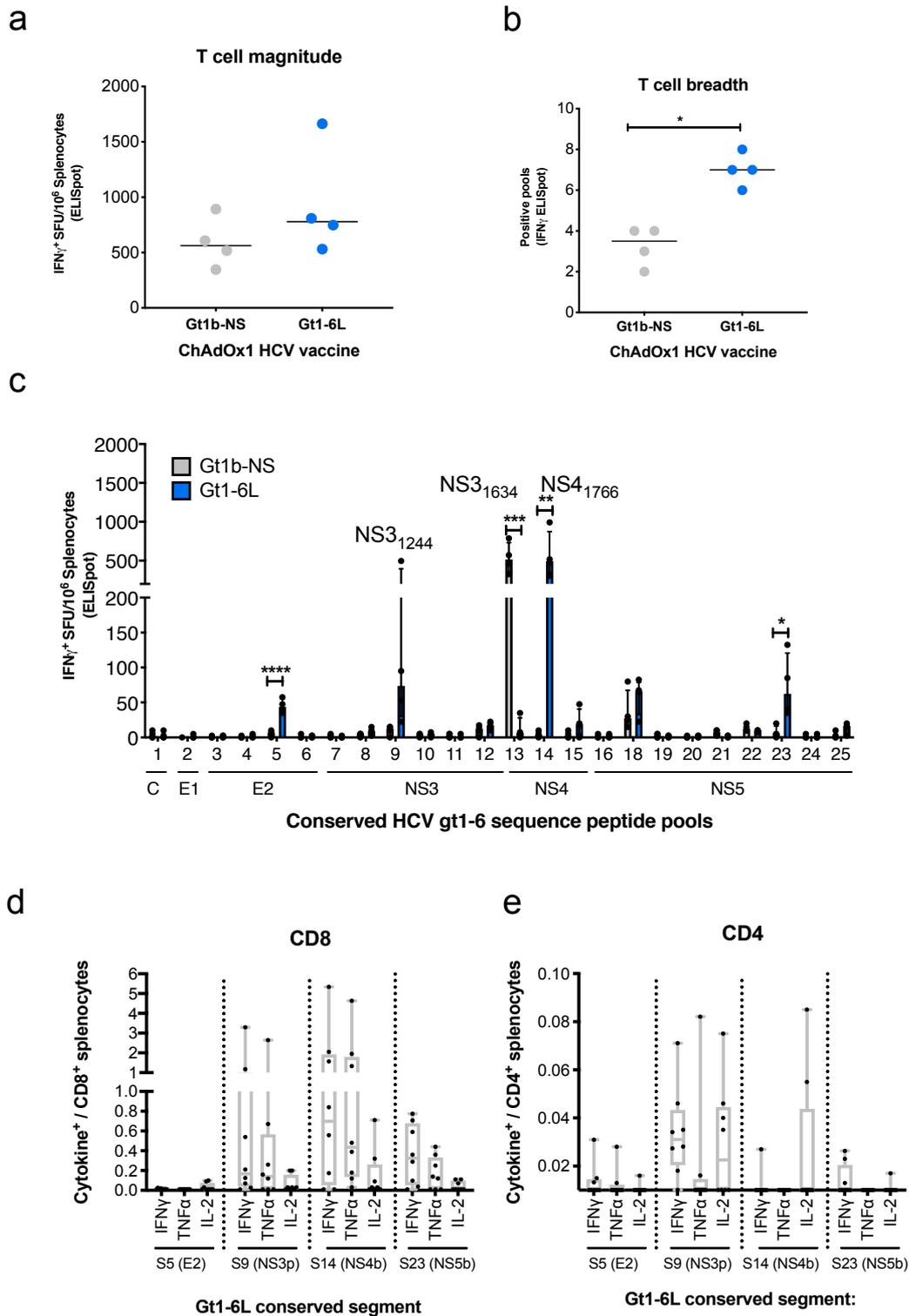
Supplementary figure 5. Comparative analysis of ChAd-Gt1b-NS and ChAd-Gt1-6 vaccine-induced immunogenicity to conserved HCV sequences: Age-matched female *C57BL/6* mice were vaccinated with 10^8 IU of either ChAd-Gt1b-NS or ChAd-gt1-6L in a 40μL intramuscular injection and harvested 3-weeks post vaccination (n=4 for each vaccine, respectively, and the experiment was

798 performed once). The total magnitude (**A**) and number of positive ELISpot peptide pools (**B**), and the

799 breadth of the vaccine-induced T cell response (**C**) to conserved gt1-6 sequence peptide pools (S1-

800 25) that cover the subtype-1b specific conserved sequence of the Gt1-6L immunogen (15-18mers

801 overlapping by 11aa). The epitopes of the highest responding peptide pools, S9 (NS3$_{1244}$) S13

802 (NS3$_{1634}$) and S14 (NS4$_{1766}$), are displayed. (**D**) Total vaccine-induced NS3$_{1244}$, NS3$_{1634}$, and NS4$_{1766}$-

803 specific cytokine producing CD8$^+$ and CD4$^+$ T cell responses are shown. Bars represent medians and

804 interquartile ranges are displayed.

Supplementary Table 1. *HLA-A*02:01*-restricted HCV-derived epitope sequences								
A2 epitope			Gt1-6L		Gt1/3		Gt1b	
Peptide sequence	Protein	H77 position	Present?	Vaccine sequence	Present?	Vaccine sequence	Present?	Vaccine sequence
YLLPRRGPRL	Core	35-44	yes	YLLPRRGPRL	yes	YLLPRRGPRL	no	-
DLMGYIPLV	Core	132-140	yes	DLMGYIPLV	yes	DLMGYIPLV	no	-
IMHTPGCV	E1	220-227	no	-	no	-	no	-
TIRRHVDLLV	E1	257-266	no	-	no	-	no	-
SMVGNWAKV	E1	363-371	no	-	no	-	no	-
RLWHYPCTI	E2	614-622	mismatch	RLWHYPCT**V**	partial	RLWHYPCT x	no	-
ALSTGLIHL	E2	684-692	yes	ALSTGLIHL	yes	ALSTGLIHL	no	-
FLLLADARV	E2	723-731	no	-	no	-	no	-
GLLGCIITSL	NS3	1038-1047	no	-	no	-	yes	GLLGCIITSL
CVNGVCWTV	NS3	1073-1081	no	-	no	-	yes	CVNGVCWTV
LLCPSGHVV	NS3	1169-1177	no	-	no	-	mismatch	LLCPSGH**A**V
ATLGFGAYM	NS3	1260-1268	yes	ATLGFGAYM	yes	ATLGFGAYM	yes	ATLGFGAYM
KLTGLGLNAV	NS3	1406-1415	no	-	no	-	mismatch	KL**S**GLG**I**NAV
YLVAYQATV	NS3	1585-1593	yes	YLVAYQATV	mismatch	YL**T**AYQATV	yes	YLVAYQATV
TLHGPTPLL	NS3	1617-1625	no	-	yes	TLHGPTPLL	yes	TLHGPTPLL
HMWNFITGI	NS4b	1764-1772	mismatch	HMWNFI**S**GI	mismatch	HMWNFI**S**GI	mismatch	HMWNFI**S**GI
SLMAFTASI	NS4b	1789-1797	mismatch	SLMAFTA**AA**	mismatch	SLMAFTA**A** x	yes	SLMAFTASI
ILAGYGAGV	NS4b	1851-1859	yes	ILAGYGAGV	yes	ILAGYGAGV	yes	ILAGYGAGV
SPDADLIEANL	NS5a	2221-2231	no	-	no	-	yes	SPDADLIEANL
ILDSFDPLR	NS5a	2252-2260	no	-	no	-	mismatch	**V**LDSFDPLR
RLIVFPDLGV	NS5b	2578-2587	no	-	no	-	yes	RLIVFPDLGV
ALYDVVSTL	NS5b	2594-2602	no	-	partial	ALYDV x x x x	yes	ALYDVVSTL
KLQDCTMLV	NS5b	2727-2735	no	-	no	-	yes	KLQDCTMLV

807

808 **Supplementary Table 1. *HLA-A*0201*-restricted HCV-derived epitope sequences**: *HLA-A*02:01*-restricted epitopes that are described in human studies

809 and reported in the Los Alamos database are displayed with corresponding sequence, respective protein, start and end position relative to the H77 reference

810 sequence, and whether the epitope is present in the HCV vaccine immunogens. The epitope sequence in the vaccine immunogen is listed as a yes for a

811 direct match, mismatch for amino acid substitution (changed residues underlined), or partial if amino acids are missing.

812

813

Supplementary table 2. Antibodies, software, repositories, and vectors			
Antibody	**Supplier**	**Cat no.**	**Clone no.**
anti-mouse anti-IFNγ mAb	Mabtech, Sweden	3321-3-250	AN18
anti-mouse IFNγ mAb R4-6A2 biotinylated	Mabtech, Sweden	3321-6-250	R4-6A2
anti-biotin alkaline phosphatase	Vector Laboratories, Burlingame, CA, USA	SP-3020	N/A
CD3-efluor450	eBioscience		17A2
CD4-AlexaFluor700	eBioscience	56-0041-82	GK1.5
CD8-peridinin chlorophyll protein (PerCP) Cy5.5	eBioscience	45-0081-82	53-6.7
IFNγ-phycoerythrin (PE)	eBioscience	12-7311-82	XMG1.2
TNFα-fluorescein isothiocyanate (FITC)	eBioscience	11-7321-41	MP6-XT22
and IL-2-oallophycocyanin (APC)	Biolegend	503810	JES6-5H4
Software name	**Manufacturer**	**Version**	
Prism	Graphpad	V8.0.1	
FlowJo	TreeStar, USA	V10.5	
Pestle	N/A	V1.8	
SPICE	NIAID, NIH	V5.35	
Name of repository	**Link**		
ICTV	https://talk.ictvonline.org/ictv_wikis/flaviviridae/w/sg_flavi/56/hcv-classification		
Vectors	**Source**		
ChAdOx1	Viral Vector Core Facility		

814