

Viral vectored hepatitis C virus vaccines generate pan-genotypic T cell responses to conserved subdominant epitopes

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- 26 1. Conserved segment HCV vaccines induce high magnitude CD4⁺ and CD8⁺ T cell responses
27 in mice.
- 28 2. Conserved segment HCV vaccines are as immunogenic as the gt1b HCV vaccine that was in
29 human trials.
- 30 3. Conserved segment HCV vaccine induced T cells target highly conserved epitopes across
31 subtypes.
- 32 4. These highly conserved epitopes are associated with spontaneous HCV resolution in
33 humans.
- 34 5. Adding the truncated shark invariant chain to the HCV immunogen increases the T cell
35 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 (sI_{tr}).

Methods: Age-matched female mice were immunised intramuscularly with ChAd (10⁸ 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 (sI_{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 hepatitis virus (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.

Very recently, preliminary results from a phase II study (ClinicalTrials.gov NCT01436357) evaluating a promising HCV T cell vaccine strategy in people who inject drugs (PWIDS) have been reported (www.niaid.nih.gov/news-events/trial-evaluating-experimental-hepatitis-c-vaccine-concludes). This approach used chimpanzee adenovirus and modified vaccinia Ankara (ChAd3 and MVA) viral vectors encoding the gt-1b specific sequence of non-structural (NS) proteins 3-5 (1985 amino acids), in a heterologous prime/boost strategy. These vectors, when used in prime/boost have been shown to be potent inducers of cellular immune responses against the encoded immunogen, in part due to an intrinsic adjuvant effect of the vectors. In spite of the high magnitude of polyfunctional CD4⁺ and CD8⁺ generated by this approach as demonstrated in early phase I human trials (26), this vaccine failed to protect PWIDS from chronic infection (27). Whilst the data indicating why this vaccine trial failed to protect people from chronic HCV infection is yet to be reported, the lack of protection highlights the need for alternative vaccine strategies.

HCV viral variability has long been recognised as a major challenge to the development of an HCV vaccine, with six common distinct HCV genotypes that are 20% divergent at the amino acid level and over one hundred genetically different subtypes worldwide (28). Although our previous data evaluating the gt-1b vaccine (reported in NCT01436357) showed evidence of T cell immune responses that were cross reactive with non-gt 1b antigens, these were reduced by more than 50% (26). Furthermore, when evaluating HCV specific T cell responses at the single epitope level we found that there was a marked reduction or absence of T cell responses against commonly circulating epitope variants both within and between HCV genotypes (29). In patients exposed to HCV, we have also shown limited cross reactivity between T cells that target gt-1 and gt-3 (30) which are the two dominant HCV genotypes globally (31). This lack of T cell cross reactivity in dominant epitopes is likely to present a major challenge to real world scenarios where multiple HCV genotypes are found circulating within the same geographical regions, and where the virus population within a host (the quasispecies) exhibits genetic variation that may rapidly escape the immune response (32). An effective vaccination strategy will need to target multiple genotypes within a target population and virus variants within an infected individual in order to overcome HCV variability and prevent viral persistence.

We therefore generated second generation HCV T cell immunogens (33), encoding conserved genomic sequence between genotype-1 and -3 (gt1/3) aiming to provide coverage for the two most dominant strains in Europe, and genotype-1, -2, -3, -4, -5 and -6 inclusive (gt1-6) to provide global coverage against all major genotypes (31). These conserved sequence immunogens consist of multiple segments of highly conserved HCV sequence across all HCV subtypes, and exclude variable HCV regions (33). We hypothesised that this approach would generate pan-genotypic T cell responses and also limit viral escape from vaccine-induced T cell immunity since mutations within conserved viral sequences are likely to carry a detrimental fitness cost (33). The gt-1-6 vaccine is particularly attractive as a global vaccine as it would best mitigate against infections from a broad range of genotypes in the current era of extensive travel and migration, reduce the risk of vaccine escape mutations, and is most attractive from a manufacturing and commercial perspective as it would focus clinical development on a single vaccine. Having previously described the rationale, development, and generation of the conserved segment vaccine candidates (ChAd-Gt1/3 and ChAd-Gt1-6; 32) we now evaluate the T cell cross-reactivity against dominant genotypes of these second generation vaccines in comparison to ChAd-Gt1b-NS. We also aim to enhance T cell immune responses against conserved regions of the HCV proteome using the truncated form of the shark invariant chain (sli_{tr}) previously shown to enhance T cell immune responses in malaria vaccine pre-clinical studies (34).

RESULTS

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

Second generation HCV T cell vaccines encoding either long (1500 amino acid immunogen; L) and short (1000 amino acid immunogen; S) HCV genomic segments conserved between (i) HCV genotypes -1 and -3 (ChAd-Gt1/3), and (ii) genotypes-1 to -6 (ChAd-Gt1-6) (**figure 1A**) were encoded in simian adenovirus vectors as previously described (ChAdOx1; 32). These vaccines (given intramuscular (IM) at 10⁸ infectious units; IU) generated high-magnitude IFN γ producing T cell response as measured in splenocytes from vaccinated *BALB/c* mice harvested 14-days post-immunisation (**figure 1B-C**). The long versions of the conserved segment immunogens (Gt1/3L and Gt1-6L), that contain all *in silico* defined conserved sequences, displayed significantly higher median

frequencies of T cells of 2390 and 2455 median spot forming unit (SFU), respectively, in *ex vivo* IFN γ ELISpot assays, compared to their respective short versions ($p = 0.0286$; **figure 1C**). The enhanced T cell response with the long version immunogen was seen across multiple antigenic genomic regions (assessed in 10 peptide pools) (**figure 1D**), predominantly targeting non-structural antigens NS3h, NS4, and NS5b.

To limit the potential immunogenicity of artificial newly formed epitopes between genomic segments that are not naturally occurring and are therefore irrelevant, short linker sequences of glycine, proline, serine, and lysine residue combinations were inserted between segments that contain *in silico* predicted strong binding epitopes (**suppl. figure 1A**). We constructed a Gt1-6L vaccine without linker sequences and showed that this generated a significantly reduced T cell response in mice compared to the Gt1-6L immunogen with linkers ($p = 0.0286$; **suppl. figure 1B-D**). We found no evidence that the T cell response generated by the vaccine was directed to the linker regions (**suppl. figure 1E**).

Conserved segment vaccines induce higher magnitude T cell responses than the ChAd-Gt1b-NS vaccine

Immunogenicity of conserved segment vaccines (ChAd-Gt1/3L and ChAd-Gt1-6L) were compared with ChAd-Gt1b-NS, a vaccine containing the full length non-structural (NS3-5) region of a genotype 1b strain (BK strain) in *BALB/c* mice (**figure 2A**). At 14-days post-immunisation, conserved segment HCV vaccines induced significantly higher frequencies of IFN γ ⁺ T cells with 2653 and 2330 median SFU/10⁶ splenocytes for ChAd-Gt1/3L ($p = 0.0422$) and ChAd-Gt1-6L ($p = 0.0294$), respectively, when compared to ChAd-Gt1b-NS induced IFN γ ⁺ T cell frequencies (699 median SFU/10⁶ splenocytes; **figure 2B**). The breadth of the immune response generated by the conserved segment vaccines was similar or higher than that induced by the ChAd-Gt1b-NS vaccine and targeted both NS and structural proteins (**figure 2C and 2D**). Vaccine-induced T cell responses were also assessed in transgenic *HLA-A*02:01* transgenic mice (**figure 2E**); with the ChAd-Gt1b-NS generating T cells that predominantly targeted the epitope, NS3₁₅₈₅₋₁₅₉₃, whilst the conserved segment vaccines predominantly targeted the E2₆₁₄₋₆₂₂ epitope suggesting that the composition of the vaccine immunogen may influence the hierarchy of T cell immune responses (immunogenic epitopes in **suppl. table 1**).

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/10⁶ splenocytes), -1b (2390 and 2486 median SFU/10⁶ splenocytes), and -3a (2609 and 1864 median SFU/10⁶ 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 IFN γ T cells specific for subtype-1b (Gt1-6L 2486 vs Gt1b-NS 664 median SFU/10⁶ splenocytes; $p < 0.0001$) and -3a compared to ChAd-Gt1b-NS (1863 vs 588 median SFU/10⁶ splenocytes, respectively; $p < 0.0001$; **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 IFN γ , TNF α , 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 NS3₁₂₄₄₋₁₂₆₀, NS4b₁₇₆₆₋₁₇₈₁, and NSb₂₇₅₆₋₂₇₇₃ > 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 (NS3₁₆₃₄ for ChAd-Gt1b-NS and NS4b₁₇₆₆ 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 (sli_{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, sli_{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, sli_{tr}, shares 24.6% sequence homology with human Ii in a

truncated form (**figure 6A**), which was previously shown to enhance immune responses to encoded antigens (34). The sli_{ir} adjuvanted vaccine demonstrated a significant increase in T cell magnitude compared to non-adjuvanted vaccine (No GA), but not when compared to a vaccine adjuvanted using the tissue plasminogen activator leading sequence (TPA-LS) that is also known to enhance T cell immune responses (35; **figure 6B-C**). The sli_{ir} adjuvanted vaccine also induced significantly broader HCV specific immune responses, targeting 19/24 conserved gt1-6L sequence segments, when compared to 6/24 conserved gt1-6L sequence segments targeted by non-adjuvanted vaccines ($p = 0.0086$; **figure 6C-D**). The sli_{ir} adjuvanted ChAd-Gt1-6L vaccine also increased the HCV genotype-1a, -1b, and -3a specific total IFN γ ELISpot response in *CD-1* outbred mice compared to the TPA-LS adjuvanted gt1-6L vaccine ($p = 0.0471$; statistically significant for gt-1a and -1b peptide stimulation **figure 6E**).

DISCUSSION

Recent efforts to generate a prophylactic vaccine against HCV have used viral vectors encoding a genotype-1b immunogen (ChAd-Gt1b-NS), generating high magnitude, broad, polyfunctional T cells when used in heterologous prime boost strategies, in healthy human volunteers (26,37). However, our previous work has also shown that some T cell responses generated by this approach target immunodominant epitopes with limited cross-reactivity to non-vaccine genotypes and a recent press release by NIH has concluded that this vaccine was not effective in preventing chronic infection in at risk PWID (27). We have therefore developed second generation HCV vaccines, ChAd-Gt1/3 and ChAd-Gt1-6, encoding HCV genomic segments that are conserved between HCV subtypes encoded in a ChAdOx1 viral vector (33) specifically designed to address the global coverage of different HCV genotypes and assessed these in pre-clinical studies.

We show that ChAd-Gt1/3 and ChAd-Gt1-6 vaccines generate HCV specific T cell responses that are of a higher magnitude than those induced by the ChAd-Gt1b-NS vaccine in inbred, outbred, and HLA-A2.1 transgenic mice. These conserved segment vaccines were designed to induce T cells against both structural and non-structural HCV antigens; in mice these T cells predominantly target non-structural HCV antigens, though T cell responses to structural antigens were also detected at low magnitude. Both CD4⁺ and CD8⁺ T cell subsets are generated from a single prime vaccination

secreting IFN γ , TNF α , and IL-2. The generation of both CD4⁺ and CD8⁺ T cells is an important criterion for the selection of vaccine candidates for human studies, since HCV resolution has been associated with the generation of CD4⁺ and CD8⁺ T cells that secrete these cytokines (7,11,23,24,38,39).

Non-structural HCV epitopes targeted by ChAd-Gt1-6 induced T cells (NS3₁₂₄₄₋₁₂₆₀, NS4₁₇₇₆₋₁₇₈₁), but not by ChAd-Gt1b-NS induced T cells, have been previously identified in the majority of acute resolving gt-1a/b, -3a, and -4 HCV infections (7,12,30,40–42). In contrast, epitopes in non-structural HCV sequence targeted by ChAd-Gt1b-NS induced T cells (NS3₁₆₂₁₋₁₆₃₇, NS5a₂₂₇₈₋₂₂₇₈, NS5b₂₄₄₇₋₂₄₇₀, NS5b₂₉₅₅₋₂₉₇₂) have been described only in a minority (~18%; 40) of resolving HCV gt-1 and -3 infections (12). Furthermore, a structural HCV epitope previously described in spontaneous resolution, E2₆₀₆₋₆₂₂ (7,12,30,43), was targeted by the ChAd-Gt1-6 induced CD4⁺ T cell response (HLA-A2 and CD-1 mice). Whilst the generation of E2 specific T cells may in theory contribute to the generation of anti-HCV antibodies (through T cell help), this vaccine is not designed to generate antibodies, and these were not evaluated. Overall, these observations from spontaneous resolvers suggest that the induction of conserved subdominant epitopes may be preferable to combat multiple HCV genotypes.

Immunodominant CD4⁺ and CD8⁺ T cell epitopes in variable viral regions display limited cross reactivity between HCV genotypes (30,42,44,45). The exclusion of variable HCV sequences containing immunodominant epitopes from an HCV immunogen may increase the targeting of subdominant epitopes by naïve T cells and therefore generate a vaccine-induced T cell response targeting subdominant epitopes that lie in conserved viral regions. Here, we demonstrate that the conserved segment vaccines generate T cells that target highly conserved subdominant epitopes (greater than 96.6% sequence homology across HCV subtypes) that are not targeted by the ChAd-Gt1b-NS vaccine, whereas the ChAd-Gt1b-NS vaccine generates T cells that target immunodominant epitopes that are not found in conserved viral regions. This result demonstrates that there is a hierarchy of immune dominance that may be manipulated through the exclusion or inclusion of particular genomic regions in rational vaccine design. This approach was also utilised in HIV vaccines 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 acting 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

demonstrated that inclusion of sli_{tr} increased the magnitude and breadth of the vaccine-induced HCV-specific T cell response. In other viral vector vaccine pre-clinical studies, sli_{tr} enhanced the immune response of viral vectors encoding malaria antigens (34). Whether sli_{tr} increases the capacity of a T cell vaccine to protect against HCV infection, remains to be shown.

In this study, we assessed a novel HCV vaccine strategy with the primary aim of inducing T cells to conserved HCV sequences. The generation of HCV antibodies, following vaccination, was not assessed since the immunogen and vaccine strategy was not designed to induce an antibody response. Our aim rather, was to generate the most potent T cell vaccine possible, that may give broad coverage against multiple HCV genotypes. We recognise that ultimately T cells alone may not protect against HCV, and in the future vaccine strategies that aim to generate both T cell and neutralising antibodies may need to be considered.

Furthermore, while our novel vaccine strategy induces T cell responses targeting conserved HCV sequences that have also been identified in spontaneous resolvers, the evaluation of vaccine efficacy is impeded by the lack of suitable small animal challenge models. While significant advances have been made in humanised animal models that are permissible to HCV infection and suitable to assess efficacious humoral immunity (49), an immunocompetent mouse model of chronic HCV infection, that can support viral replication, to assess vaccine-induced protective T cell responses is not readily available. Future efforts to develop a readily accessible, immunocompetent small animal model of chronic HCV infection should be prioritised. Based on the data presented here, ChAd-Gt1-6L should be the focus of future challenge studies and clinical trials in order to advance a single HCV vaccine for global use through the clinical pipeline to be available to those who need it.

MATERIALS AND METHODS

Vaccine nomenclature

The ChAdOx1 conserved segment HCV T cell vaccines encode the conserved HCV sequence segment of (1) genotype-1 and -3 subtypes and (2) all subtypes in genotype-1 to -6 as previously described (33). They are referred to here as '*ChAd-Gt1/3*' and '*ChAd-Gt1-6*'. Both vaccines have long and short immunogen versions, i.e. the shorter gt1/3 immunogen of 1000 amino acids is referred to as

'ChAd-Gt1/3S'. The longer gt1/3 immunogen of 1500 amino acids is referred to as 'ChAd-Gt1/3L'. The first-generation HCV T cell vaccine that encodes the genotype-1b non-structural sequence (NS3-5) is referred to as ChAd-Gt1b-NS. A ChAd encoding the eGFP protein sequence was used as a vehicle control. The conserved segment Gt1-6L vaccine without linkers between genomic segments is referred to as ChAd-Gt1-6L_NL. Genetic adjuvants are described using suffixes on vaccine names, for example, the shark invariant chain is ChAd-Gt1-6L-sli.

Animal experiments

All mouse studies were performed at the Biomedical Services Building (BSB), Oxford, according to UK Home Office Regulations (project license numbers 30/2744 and P874AC0FO) and approved by the local ethical review board at the University of Oxford. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the UK Animals (Scientific Procedure) Act, 1986. Groups of four to eight age-matched 6-8 week old female mice (BALB/c, C57BL/6, CD-1, *HLA-A*02:01* transgenic mice) were used throughout and housed at a pathogen free facility in individually-vented cages and fed a commercial block nutrient diet (Harlan Teklad Lab Blocks). Inbred strains (the same H-2 haplotype, e.g. H-2K/D^b in C57BL/6 mice) were used to ensure limited immune response variance between individual subjects in the same group. The outbred strain, CD-1, was used to detect differences in the broad range of epitopes targeted by vaccine-induced T cell responses (which may not be detected in inbred strains). *HLA-A*0201* transgenic mice were used to assess the immunogenicity of conserved HCV epitopes when presented by human major histocompatibility complex (MHC) receptors, to indicate if these vaccines may be immunogenic in humans. After a 1-week adaptation period after arrival at the animal facility, mice were vaccinated intramuscularly (IM, 26G needle) in the left quadricep with 40μL of viral vector vaccine solution (10⁸ infectious units of vaccine in sterile PBS, immunised in the afternoon). Mice were harvested either 2- or 3-weeks post vaccination by schedule 1 (CO₂ exposure followed by cervical dislocation).

Peptides

Peptides were obtained through BEI Resources, NIAID, NIH (genotype-1a H77, genotype-1b J4, genotype-3a K3a650). These peptides were HCV genotype-specific 15-18mer synthetic peptides, overlapping by 11 amino acids, and covering the length of the HCV proteome (optimal for CD4 and

CD8 T cell activation). Peptides were initially dissolved in dimethyl sulfoxide (DMSO) at 40mg/mL and subsequently pooled into 10 pools at 300µg/mL labelled A (core), B (E1), C (E2), D (NS2), F (NS3p, protease), G (NS3h, helicase), H (NS4), I (NS5a), L, (NS5bl, amino acids 2421-2718), and M (NS5bll, amino acids 2719-3011). Peptide minipools (e.g. H1-H6), segment pools (S1-S25 matching conserved Gt1-6L sequence segments), and individual peptides containing *HLA-A*02:01* epitopes (described in human studies and reported in the Los Alamos database) were generated to stimulate splenocytes in ELISpot assays.

Splenocyte isolation

Harvested mouse splenocytes were harvested immediately after schedule 1 killing (CO₂ exposure followed by cervical dislocation) and collected in ice cold PBS. Lymphocytes were isolated by mechanical processing using a sterile plunger and 40µm cell strainer. Red blood cells were lysed with ACK lysis buffer for no longer than one minute and remaining cells resuspended in R10 media (RPMI 1640 media with L-glutamine (5%), penicillin-streptomycin (5%), and 10% foetal calf serum). Cell yields were calculated using a Guava Personal Cell analysis system (Merck Millipore 0100-14230) and the Muse® Cell Analyser (Merck Millipore). The machine was calibrated prior to cell counting using Guava check beads (16-0040).

Ex vivo IFN γ ELISpots

Multiscreen®_{HTS} IP filter plates (PVDF; Merck Millipore) were pre-wetted with 20µL of 35% ethanol per well for no longer than 60 seconds. Plates were washed with PBS and pre-coated with anti-mouse anti-IFN γ mAb (AN18, 0.5µg/well, 1:200 dilution, Mabtech, Sweden) overnight, then washed and blocked with R10 for two hours at 37°C. After blocking, cells were plated at 1-2x10⁵ cells per well in 50µL R10 media and stimulated for 20-24 hours at 37°C with 50µL HCV genotype-1a, -1b, -3a peptide pools, minipools, or peptides (3µg/mL final peptide concentration in 100µL total R10 media; NIH, MD, USA), a DMSO negative control without HCV peptides to measure background IFN γ ⁺ SFU responses and a concanavalin positive control (conA, 10µg, Sigma). Bound IFN γ was detected using anti-mouse IFN γ mAb R4-6A2 biotinylated (1:2000 dilution, Mabtech, Sweden), anti-biotin alkaline phosphatase (1:750, Vector Laboratories, Burlingame, CA, USA), and BCIP/NBT phosphatase substrate (Thermo Scientific, IL, USA). T cell responses are reported as IFN γ ⁺ SFU/10⁶ splenocytes and

the total T cell magnitude is the sum SFU of the positive individual peptide pools minus the mean DMSO SFU multiplied by the number of positive peptide pools. Peptide pools are considered positive when greater than the mean of the DMSO negative control plus three standard deviations. Antibody details can be found in the **supplementary table 2**.

Intracellular cytokine staining

Splenocytes were stimulated using HCV genotype-1a, -1b, and -3a peptide pool combinations (A+B+C = Core, E1, E2; F+G+M = NS3-4, I+L+M = NS5a-b, 1.5µg/mL, 15-18mers overlapping by 11 amino acids). A negative control (DMSO) and PMA (phorbol 12-myristate 13-acetate)/ionomycin positive control (50 and 500ng/mL, respectively) were used. Cells were stimulated for 6 hours with peptide pools (4µg/mL GolgiPlug™ (BD Biosciences) was added for the last 4 hours of the stimulation). Cells were stained with fixable Near-IR live/dead dye (Life Technologies, USA), CD3-eFluor450, CD4-AlexaFluor700, CD8-peridinin chlorophyll protein (PerCP) Cy5.5 for 30 minutes at 4°C, before being fixed and permeabilised with fixation/permeabilization solution (BD Biosciences) at 4°C for 10 minutes. Following fixation, cells were stained with IFNγ-phycoerythrin (PE), TNFα-fluorescein isothiocyanate (FITC), and IL-2-oallophycocyanin (APC) at 4°C for 30 minutes, and subsequently washed and run on the LSRII flow cytometer. ICS data was corrected for background by subtracting the cytokine production as a percentage of CD4⁺ or CD8⁺ T cell subsets in a matched DMSO negative control. Gating and analysis were performed in FlowJo (TreeStar, v10.5, USA). FlowJo Boolean gating was used for cytokine co-expression and graphs produced in Pestle (v1.8), and SPICE (NIAID, NIH, v5.35). Antibody details can be found in the **supplementary table 2**.

Sequence analysis

HCV amino acid sequences were aligned and analysed in Aliview (version 1.18). HCV subtype amino acid sequences (International Committee on the Taxonomy of Viruses, May 2019) were obtained from UniProt.org. Basic Local Alignment Search Tool (BLAST) analysis was done using the protein BLAST tool (NCBI; National Centre for Biotechnology Information website).

Statistical analysis

Data were analysed using GraphPad prism (version 8.0.1). Preliminary studies were undertaken to determine appropriate sample sizes. The D'Agostino and Pearson test was used to determine data distribution normality. Unless otherwise stated, non-parametric tests (Mann Whitney or Kruskal-Wallis test) were used to determine significant difference between two group medians at 95% confidence intervals between two or more groups, respectively. *P* values less than 0.05 indicate a significant difference: $p < 0.05 = *$, $<0.01 = **$, $<0.001 = ***$, and $<0.0001 = ****$. Only statistical differences (asterisks) are displayed.

AUTHOR CONTRIBUTIONS

Timothy Donnison: Methodology, Conceptualisation, Investigation, Formal analysis, Visualisation, Project administration, Writing – Original Draft. **Anette von Delft:** Conceptualisation, Writing – Review & Editing. **Anthony Brown:** Methodology, Resources, Investigation. **Leo Swadling:** Investigation, Writing – Review & Editing. **Claire Hutchings:** Methodology, Resources, Investigation, Writing – Review & Editing. **Tomáš Hanké:** Conceptualisation, Writing – Review & Editing. **Senthil Chinnakannan:** Supervision, Resources, Investigation, Writing – Review & Editing. **Eleanor Barnes:** Funding acquisition, Project administration, Supervision, Writing – Review & Editing.

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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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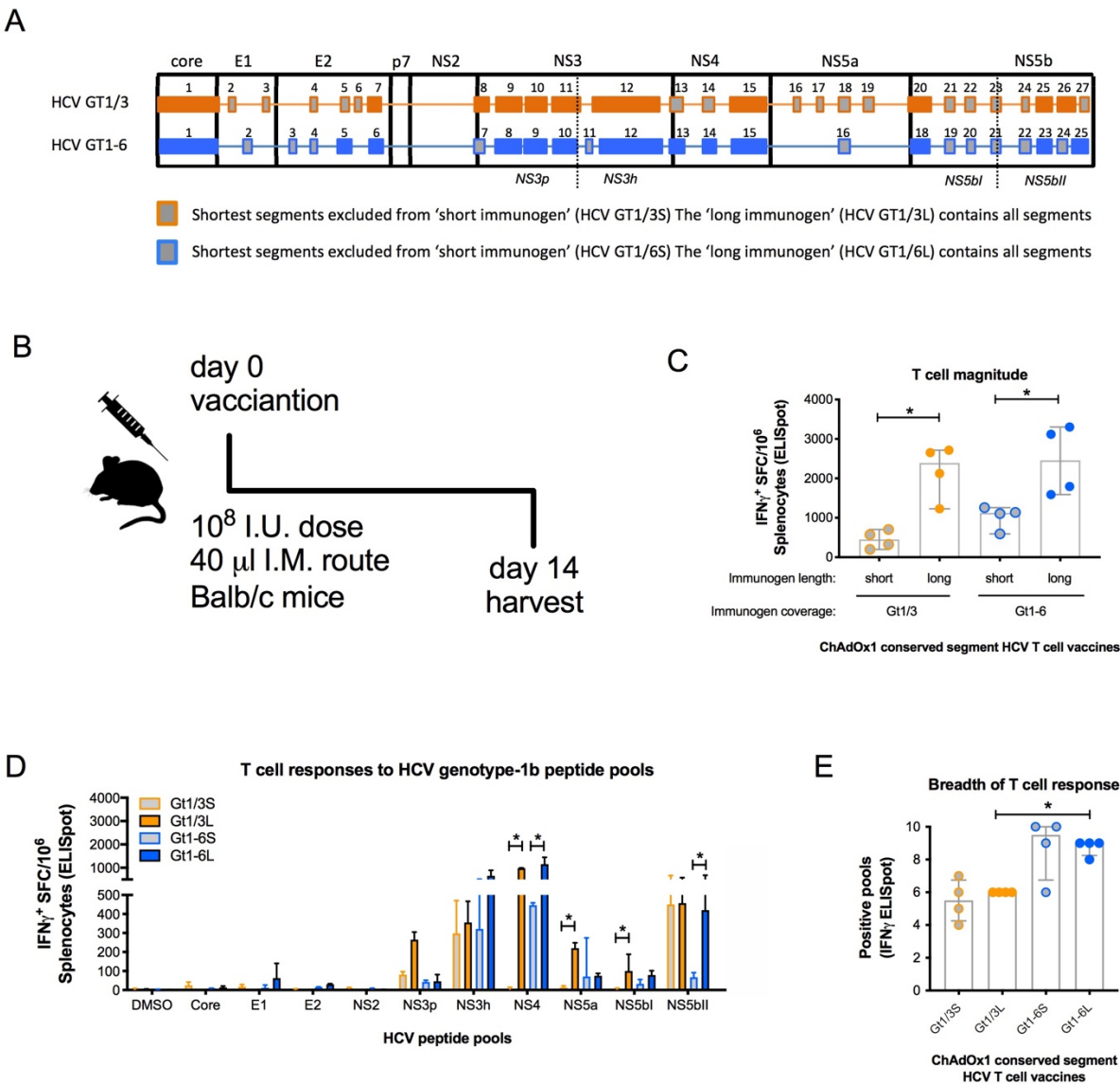
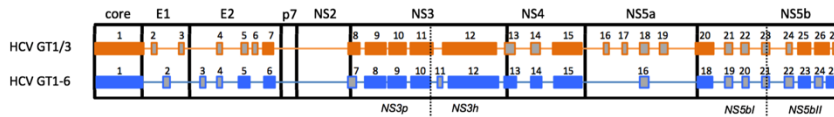


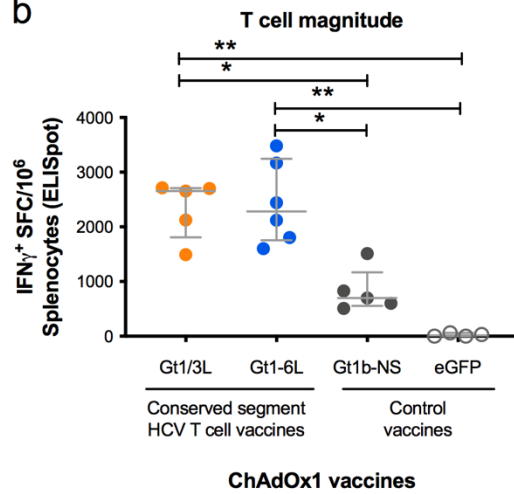
Figure 1. Immunogen design, in vitro expression and in vivo vaccine immunogenicity of conserved segment HCV T cell vaccines: (A) Conserved segment HCV T cell immunogens that contain gene segments that correspond to conserved viral sequences across viral genotypes 1 and 3, and 1 to 6. Segments are numbered 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 IFN γ -producing

639 SFU/10⁶ 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/10⁶ 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.

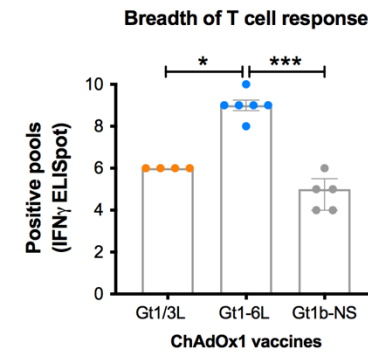
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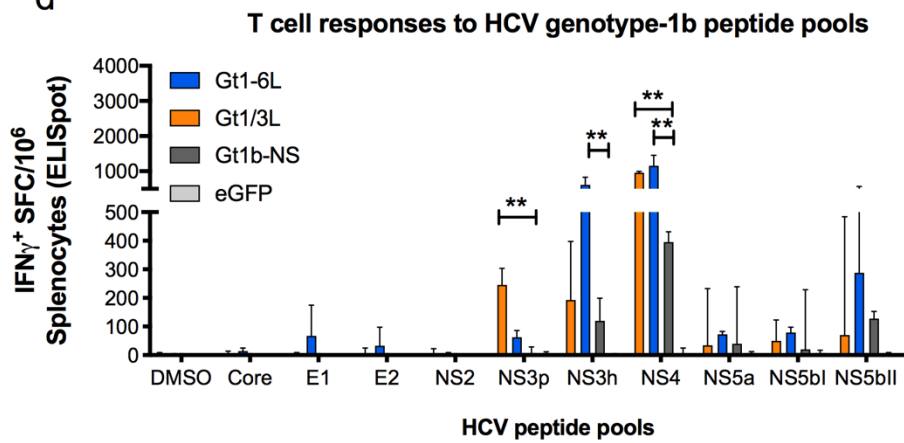
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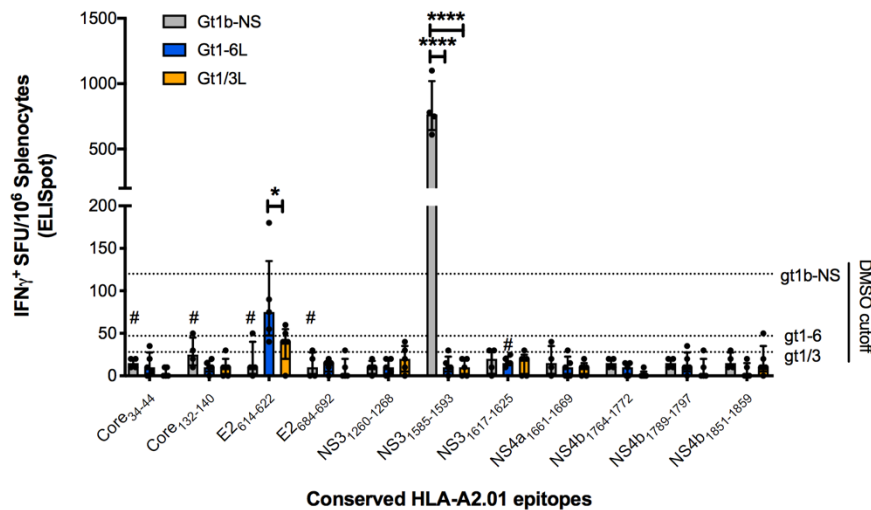


Figure 2. Comparative assessment of vaccine-induced T cell responses between conserved segment HCV T cell vaccines and subtype-1b HCV T cell vaccine: HCV T cell vaccine immunogen design (**A**). The ex vivo IFN γ ELISpot assay response for vaccine-induced T cell magnitude (**B**), breadth by number of positive peptide pools (**C**), and T cell magnitude to individual peptide pools across the full length of the HCV proteome (15-18mers overlapping by 11aa; **D**). Four to six female age matched *BALB/c* mice were vaccinated per group, each mouse receiving 10⁸ IU of vaccine in a 40 μ L intramuscular injection and harvested 14 days post-vaccination. Bars represent the median SFU/10⁶ splenocytes, with interquartile range displayed. Data is combination of two experiments. (**E**) The IFN γ ELISpot response to previously identified HLA-A*0201 restricted HCV epitopes in *HLA-A*02:01* transgenic mice (5 mice/group received 10⁸ IU single intramuscular immunisation and were harvested 14 days post-vaccination) stimulated with genotype-1b peptides (15-18mer) containing *HLA-A*02:01* identified epitopes described in human studies and reported in the Los Alamos database. Hashes indicate epitopes that are not present in the vaccine immunogen. The experiment was performed once. Pools in all experiments are defined as positive when greater than the mean of the DMSO negative control plus three standard deviations. Bars represent the median SFU/10⁶ 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**, <0.001***, <0.0001****. Only statistically significant differences between groups, indicated by an asterisk, are shown.

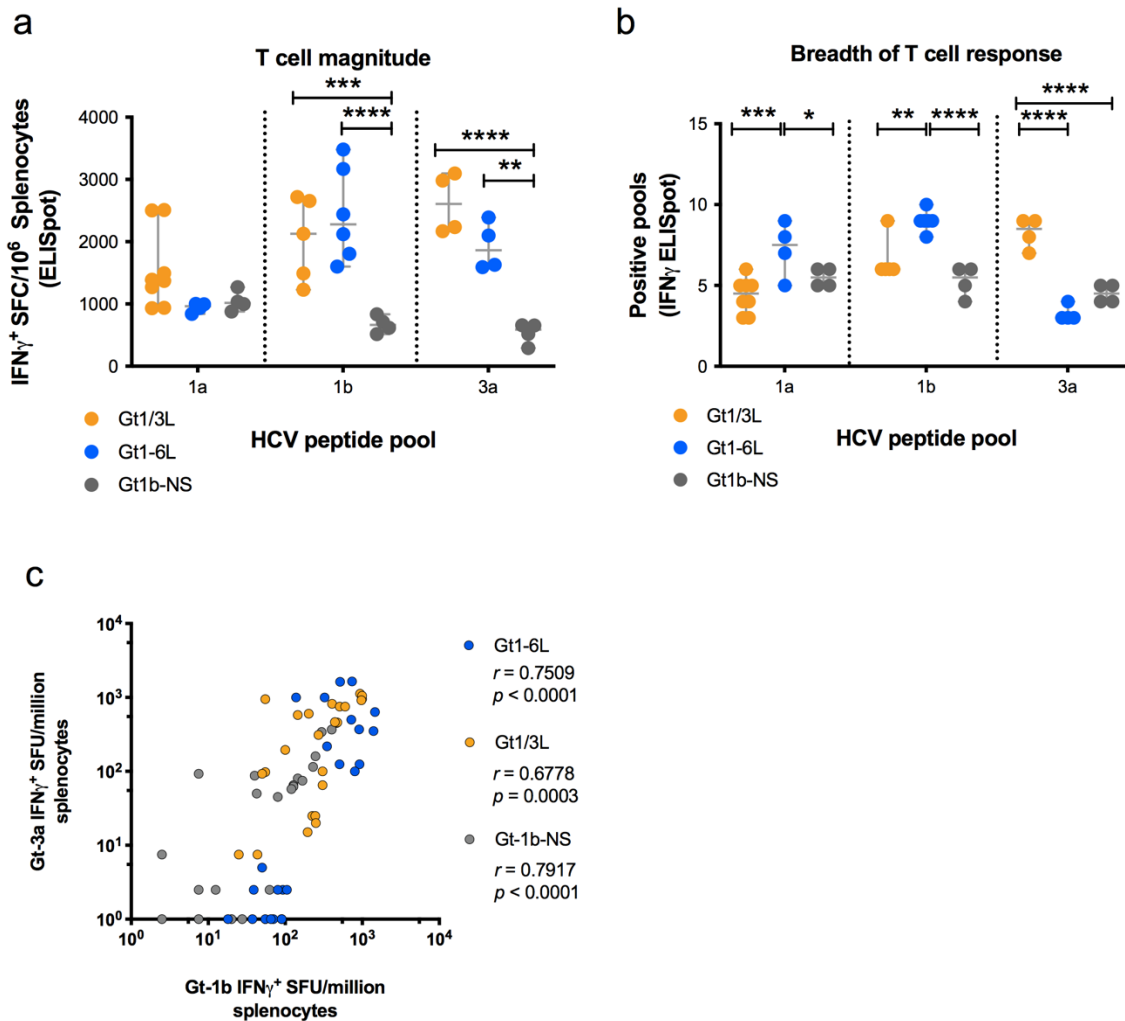


Figure 3. Intergenotypic T cell responses induced by HCV T cell vaccines: Splenocytes from age-matched female *BALB/c* mice (n=4-8/group) that received a single 10⁸ IU vaccine dose in a 40 μ L intramuscular immunisation with three different HCV T cell ChAd vaccines (gt-1b-NS, gt1-6L, gt1/3) were harvested 14 days post-vaccination and stimulated with genotype-1a (H77), -1b (J4), and -3a (k3a650) peptides in 10 pools and IFN γ producing cells were detected by *ex vivo* IFN γ 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/10⁶ 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.

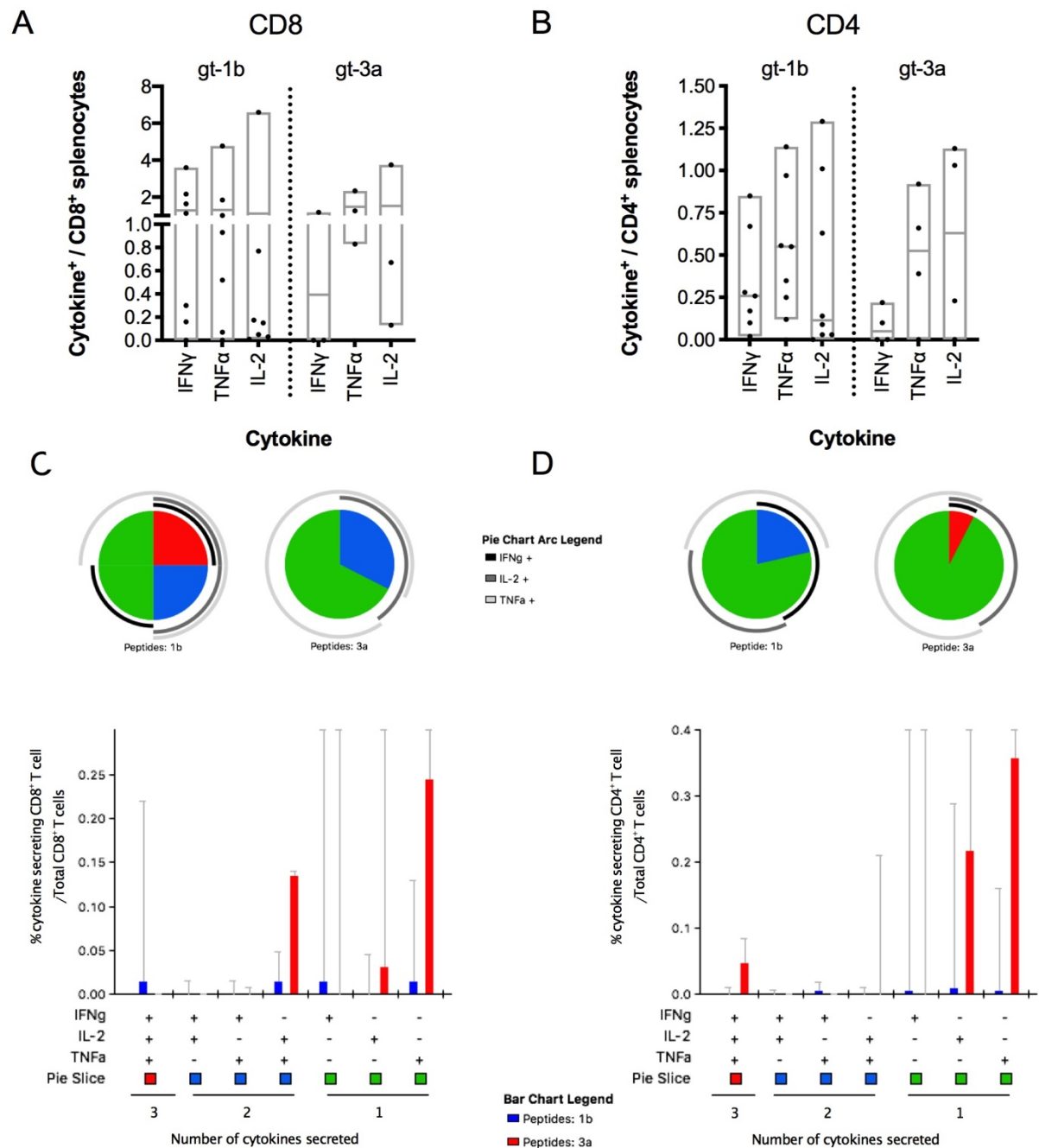


Figure 4. Functionality of vaccine-induced HCV genotype-1b and -3a specific T cell responses:

Intracellular staining of IFN γ , TNF α , and IL-2 produced by splenic CD4⁺ and CD8⁺ T cells from age-matched female C57BL/6 mice immunised with 10⁸ 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 IFN γ , TNF α , 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.

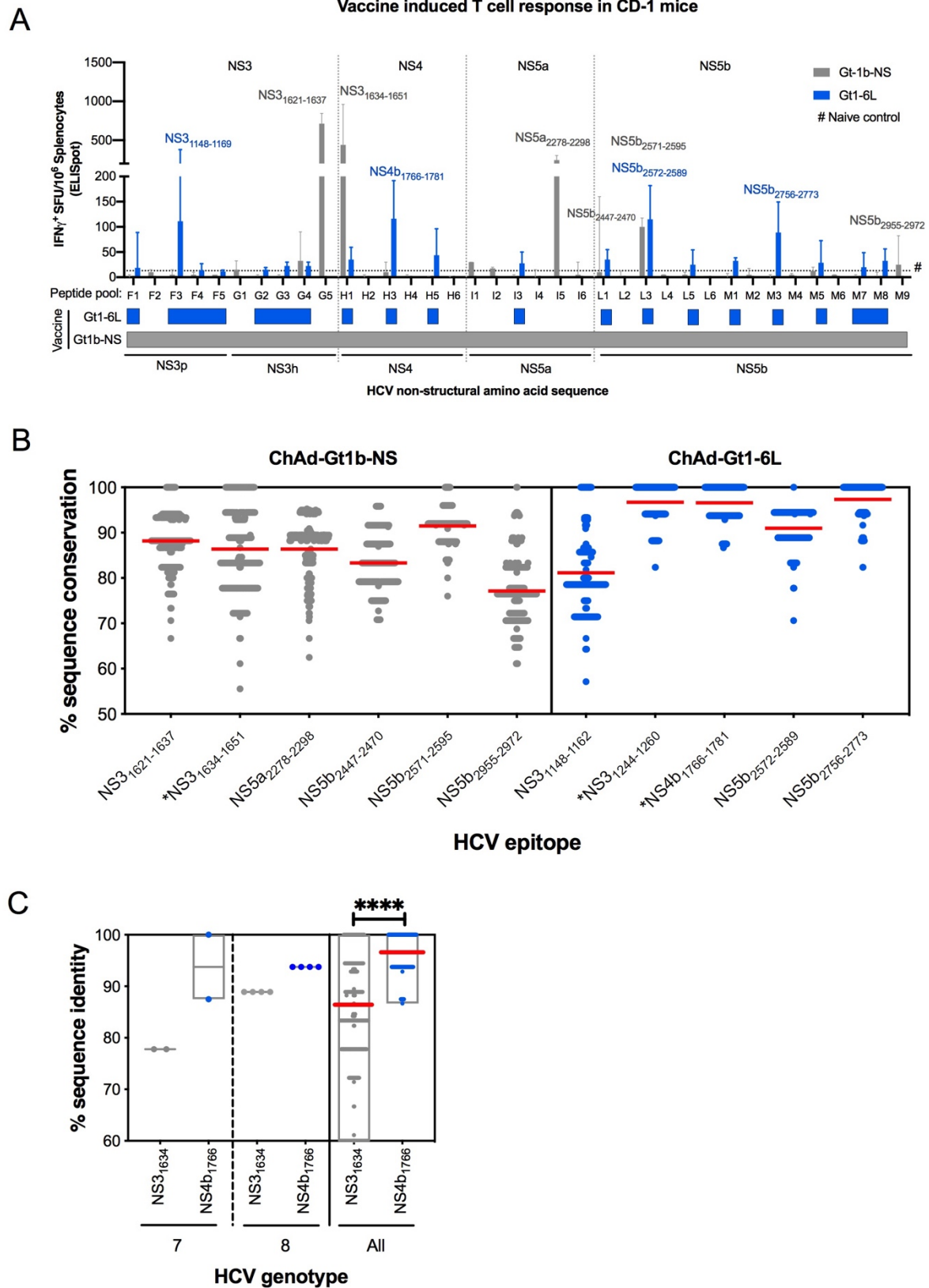


Figure 5. Comparative analysis of ChAdOx1-gt1b-NS and ChAdOx1-gt1-6 vaccine-induced immunogenicity to conserved HCV epitopes: Age-matched female *CD-1* mice were vaccinated

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 NS3₁₆₃₄ and NS4₁₇₆₆ 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.

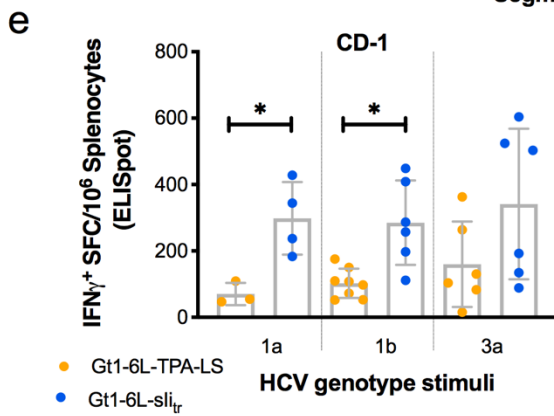
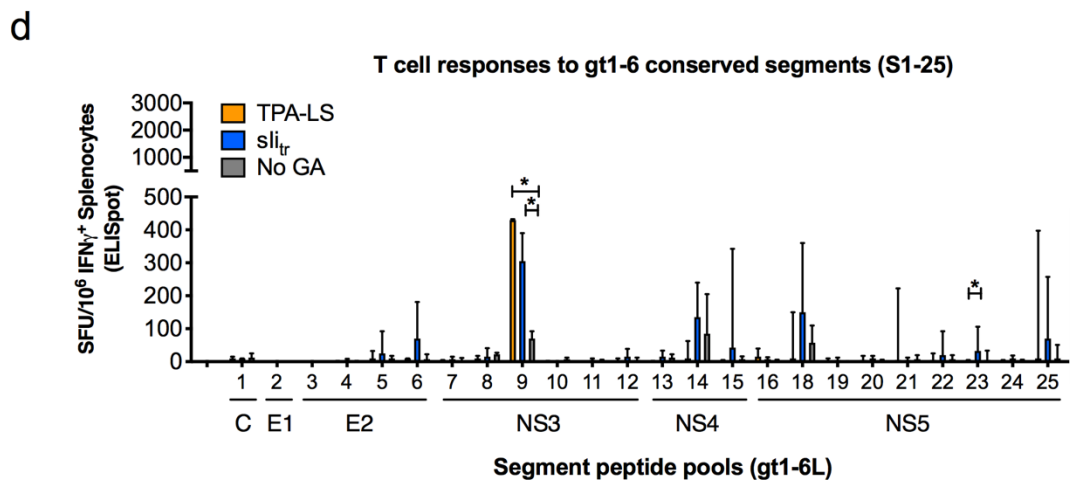
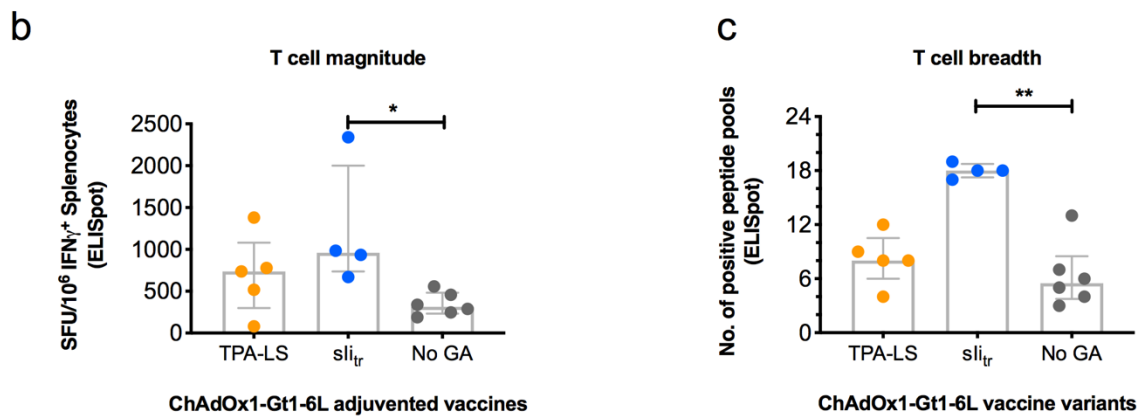
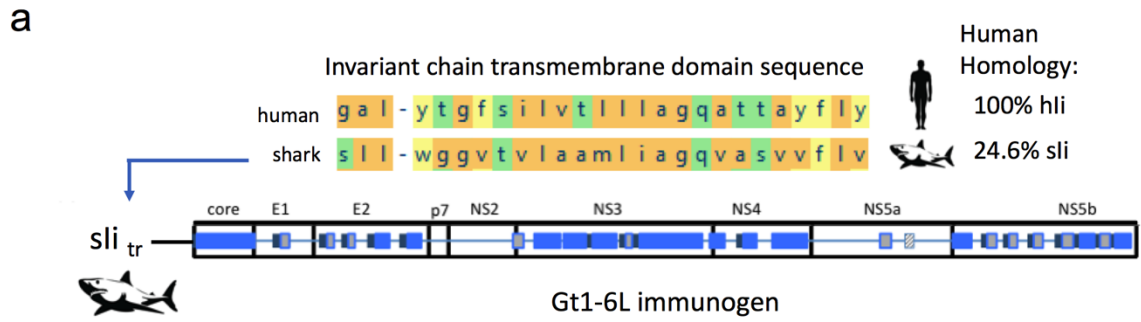


Figure 6. The design of HCV viral vector vaccines with genetic adjuvant truncated shark invariant chain (sli_{tr}) and vaccine immunogenicity in C57BL/6 and CD-1 mice: (A) A schematic of the truncated shark invariant chain (sli_{tr}) sequence and truncated human invariant chain sequence alignment (hli_{tr}). 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 10⁸ 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 IFN_γ 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 10⁷ IU IM ChAdOx1-Gt1-6L vaccines. Bars represent the median SFU/10⁶ 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.

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)	YAAQGYKVLVLPNSVAA	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)	LRAFTEAMTRYSPPGDP	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)	SNSLLRHHNMVYATTSRASLRQK	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)	KLTPIPAASQLDLGWFV	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

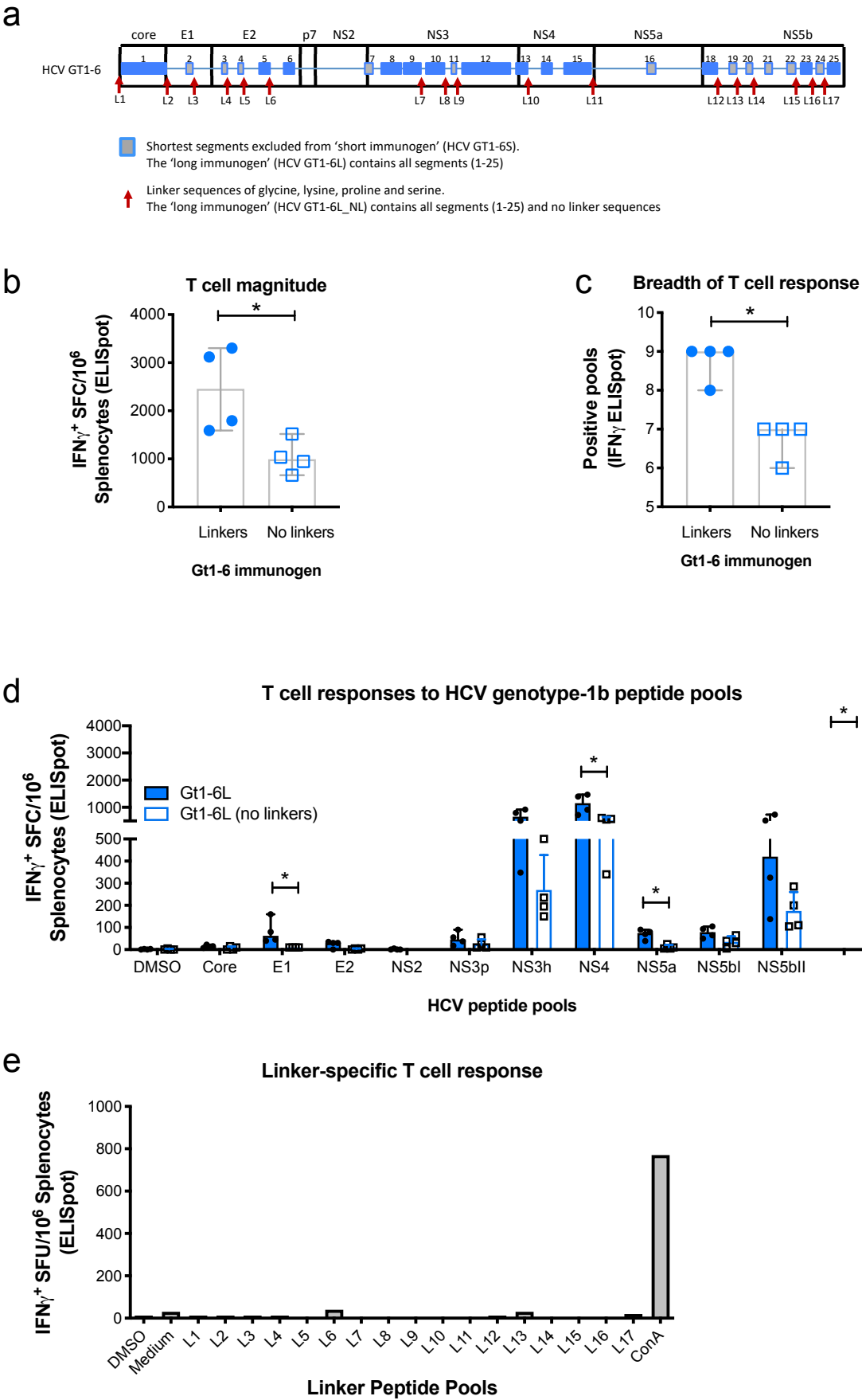
738 Supplementary figure 4. Comparative analysis of T cell HCV epitopes across genotype-1 to -6

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

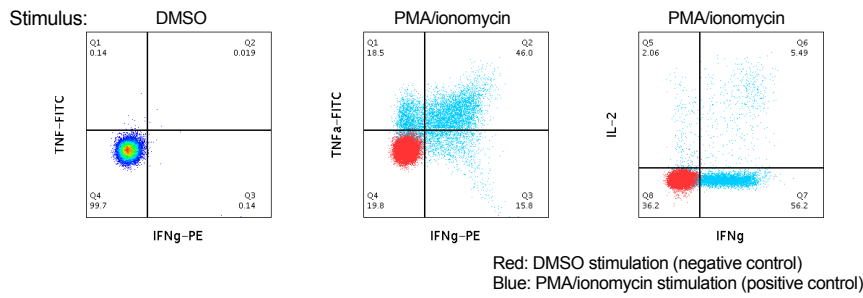
742 Supplementary table 2. Antibodies, software, repositories, and vectors

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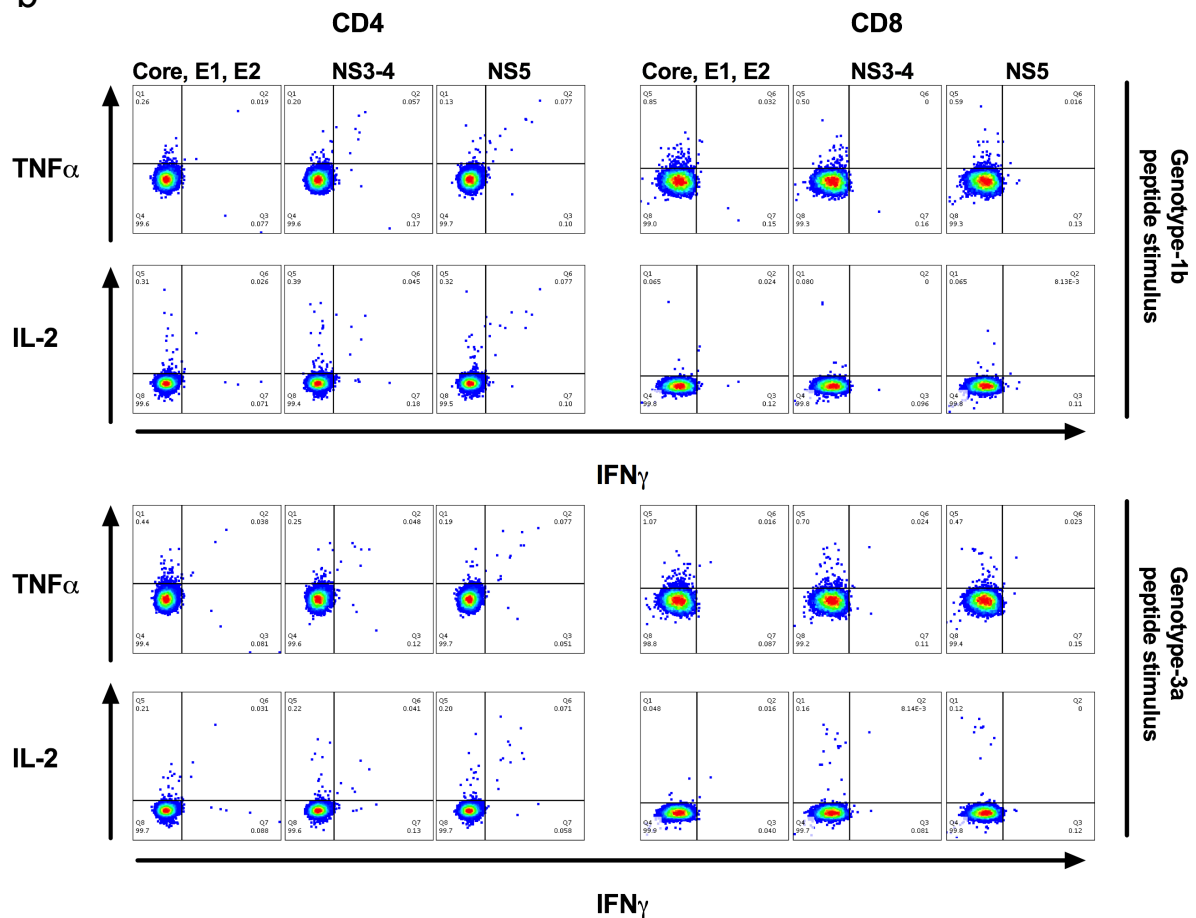


Supplementary figure 1. The effect of linker sequences between gt1-6 gene segments on vaccine-induced immunogenicity: The conserved segment vaccine, ChAd-gt1-6, with linker sequences. Red arrows indicate the location of all 17 linkers (L1-17) that were inserted between conserved gt1-6 gene segments (**A**). The *ex vivo* IFN γ ELISpot assay for vaccine-induced T cell magnitude (**B**), breadth indicated by number of positive peptide pools (**C**), T cell magnitude of individual peptide pools (**D**), and vaccine-induced immunogenicity to linker sequences in junction regions between HCV gene segments (**E**). DMSO, medium (R10), and Concanavalin A (ConA) were used as two negative controls and a positive control, respectively. T cell magnitude is the total of the positive individual peptide pools. Pools are considered positive when greater than the mean of the DMSO negative control plus three standard deviations. Four female age-matched *BALB/c* mice were vaccinated per group, each mouse receiving 10^8 IU of ChAd-gt1-6L in a 40 μ L intramuscular injection and harvested 14 days post-vaccination. Bars represent the median SFU/ 10^6 splenocytes, with interquartile range displayed. Mann Whitney tests 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**, <0.001***, <0.0001****.

a



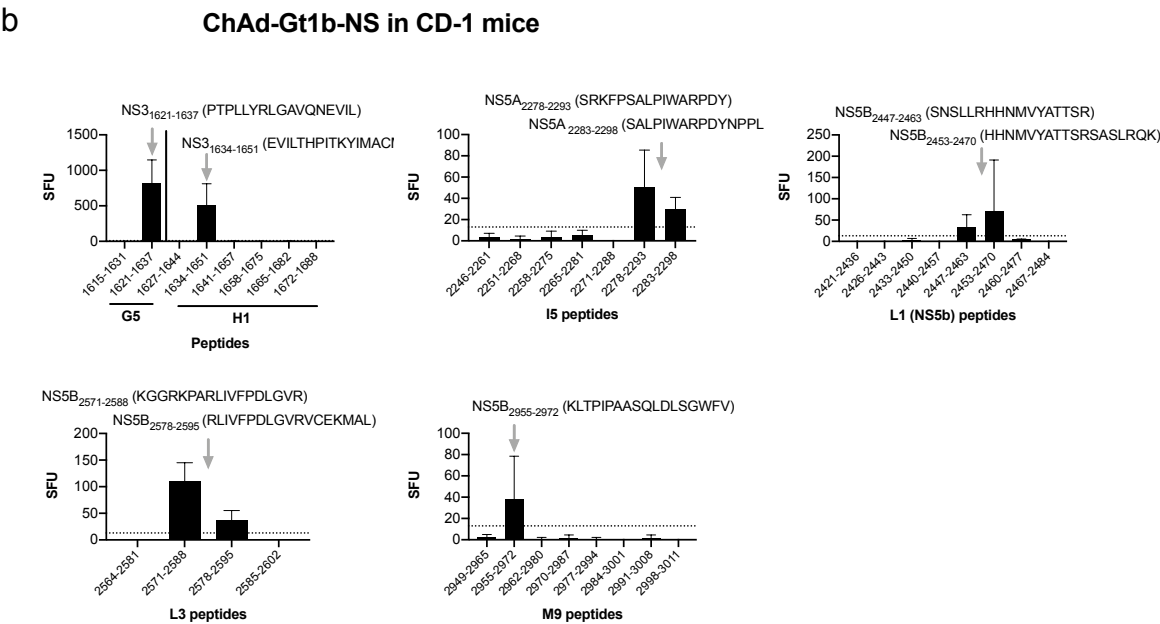
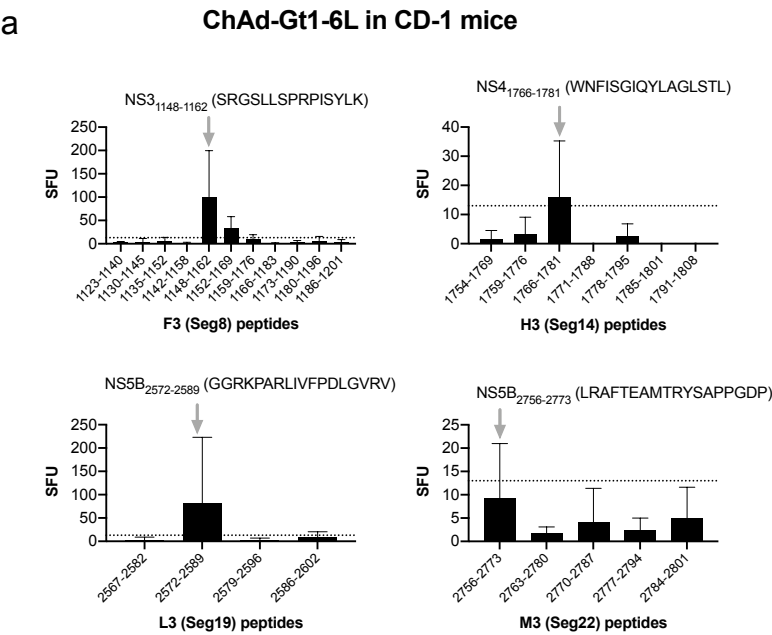
b



Supplementary figure 2. Flow cytometry plots of intracellularly stained murine splenocytes: (A) Gating strategy to identify CD4⁺ and CD8⁺ T cell subset cytokine production using DMSO (negative) and PMA/ionomycin (positive) controls. **(B)** Example plots of 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 10⁸ 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).

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773

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

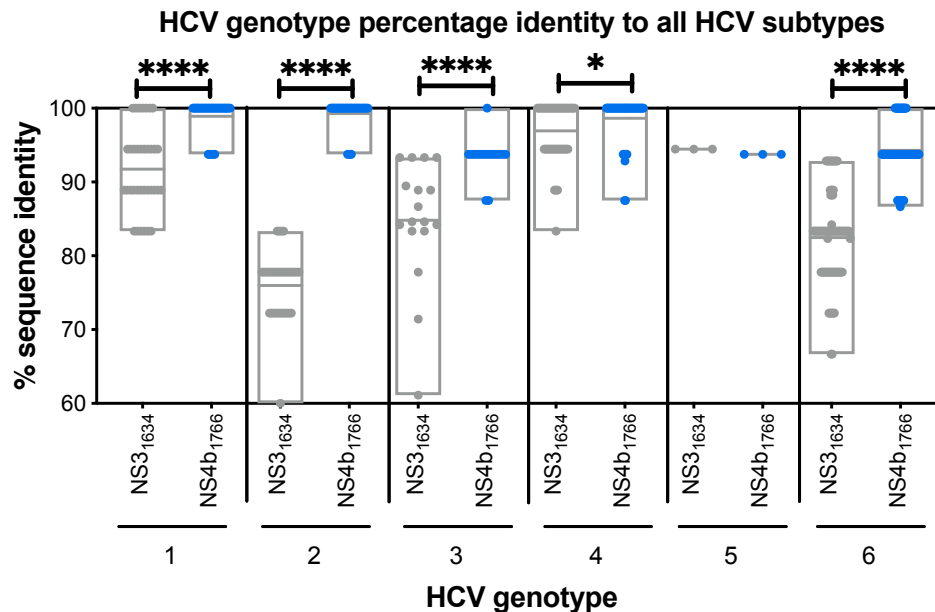
775 Groups of 4 age-matched female CD-1 mice were vaccinated with 10⁸ IU of vaccine in a 40uL

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

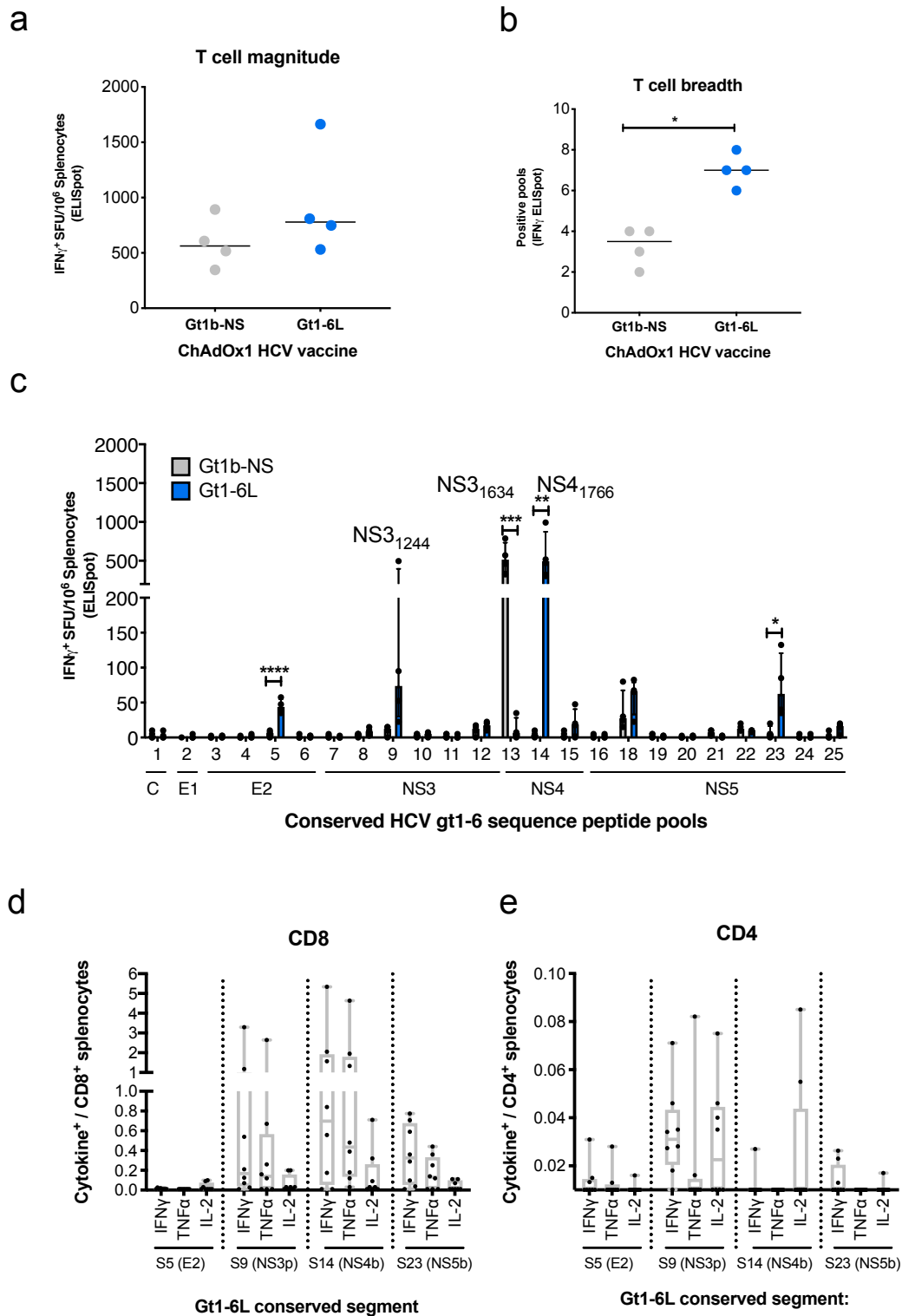
778 splenocytes stimulated with individual peptides of positive peptide minipools. Pools are considered

positive when greater than the mean of the DMSO negative control plus three standard deviations and greater than the *CD-1* naïve unvaccinated control ELISpot response. Peptides are 15-18mers, overlap by 11aa, and cover the genotype-1b specific NS proteome. Bars represent the median SFU/10⁶ splenocytes, with interquartile range displayed.



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

The percentage sequence identity when comparing NS3₁₆₃₄ and NS4₁₇₆₆ epitope sequences across HCV genotypes 1 to 6 with the epitope sequence in each vaccine (each HCV subtype is a data point) with means and ranges displayed (subtypes listed and defined by the International Committee for the Taxonomy of Viruses [ICTV] as of May 2019). The NS3₁₆₃₄ epitope was targeted by the ChAd-Gt1b-NS vaccine whereas the NS4₁₇₆₆ epitope was targeted by the ChAd-Gt1-6L vaccine. Mann Whitney tests 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**, <0.001***, <0.0001****.



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 10⁸ IU of either ChAd-Gt1b-NS or ChAd-gt1-6L in a 40 μ L 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 (NS3₁₂₄₄) S13
802 (NS3₁₆₃₄) and S14 (NS4₁₇₆₆), are displayed. (**D**) Total vaccine-induced NS3₁₂₄₄, NS3₁₆₃₄, and NS4₁₇₆₆-
803 specific cytokine producing CD8⁺ and CD4⁺ T cell responses are shown. Bars represent medians and
804 interquartile ranges are displayed.

Supplementary Table 1. <i>HLA-A*02:01</i> -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
YLLPRRGPRRL	Core	35-44	yes	YLLPRRGPRRL	yes	YLLPRRGPRRL	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	RLWHYPCTV	partial	RLWHYPCT x	no	-
ALSTGLIHL	E2	684-692	yes	ALSTGLIHL	yes	ALSTGLIHL	no	-
FLLADARV	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	LLCPSGHAV
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 I 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	SLMAFTAA A	mismatch	SLMAFTAA 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-IFN γ mAb	Mabtech, Sweden	3321-3-250	AN18
anti-mouse IFN γ 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
IFN γ -phycoerythrin (PE)	eBioscience	12-7311-82	XMG1.2
TNF α -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		