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A Human Vaccine Strategy Based On Chimpanzee Adenoviral and MVA Vectors That Primes, Boosts and Sustains Functional HCV Specific T-Cell Memory*

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Abstract

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Competing interests: S. Colloca, A.F., R.C., and A.N. are named inventors on patent applications covering HCV-vectored vaccines and chimpanzee adenovirus vectors [WO 2006133911 (A3) hepatitis C virus nucleic acid vaccine, WO 2005071093 (A3) chimpanzee adenovirus vaccine carriers, WO 03031588 (A2) hepatitis C virus vaccine]. P.K. has acted as a consultant to Tibotec and Pfizer on antiviral therapy. The other authors declare that they have no competing interests.

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A protective vaccine against hepatitis C virus (HCV) remains an unmet clinical need. HCV infects millions of people worldwide and is a leading cause of liver cirrhosis and hepatocellular cancer. Animal challenge experiments, immunogenetics studies and assessment of host immunity during acute infection highlight the critical role that effective T-cell immunity plays in viral control. In this first-in-man study we have induced antiviral immunity with functional characteristics analogous to those associated with viral control in natural infection, and improved upon a vaccine based on adenoviral vectors alone. We assessed a heterologous prime-boost vaccination strategy based on a replicative defective simian adenoviral vector (ChAd3) and modified vaccinia Ankara (MVA) vector encoding the NS3, NS4, NS5A and NS5B proteins of HCV genotype-1b.

Analysis employed single cell mass cytometry (CyTOF), and HLA class-I peptide tetramer technology in healthy human volunteers. We show that HCV specific T-cells induced by ChAd3 are optimally boosted with MVA, and generate very high levels of both CD8+ and CD4+ HCV specific T-cells targeting multiple HCV antigens. Sustained memory and effector T-cell populations are generated and T-cell memory evolved over time with improvement of quality (proliferation and polyfunctionality) following heterologous MVA boost.

We have developed a HCV vaccine strategy, with durable, broad, sustained and balanced T-cell responses, characteristic of those associated with viral control, paving the way for the first efficacy studies of a prophylactic HCV vaccine.

Keywords

Hepatitis C Virus; HCV; Hepatitis; Vaccine; Adenoviral; modified vaccinia Ankara; Prophylactic

Introduction

Hepatitis C virus (HCV) infection is a leading cause of liver cirrhosis and hepatocellular cancer, with millions of people afflicted worldwide(1). Although new oral antivirals are available (Reviewed in: (2, 3)), representing a real advance in the field, these are unaffordable and unavailable to most people, least effective in patients with advanced liver disease, associated with the development of viral resistance, and do not provide protection from reinfection(4). For these reasons an effective vaccine to prevent chronic infection remains of clinical importance.

After primary infection a proportion of those infected spontaneously resolve infection, leading to viral eradication and effectively representing long-term clinical cure(1, 5). Therefore, an effective vaccine against HCV would not need to provide sterilising immunity, but would aim to recapitulate or accelerate the immune pathway followed in natural infection to prevent disease chronicity.

HCV may be particularly susceptible to a T-cell vaccination strategy(6). Although the correlates of protection in HCV are imperfectly defined, studies of host genetic and antiviral immune responses have shown that T-cells play a critical role in viral control during primary infection. This is evidenced by; associations of class-I Human Leucocyte Antigens (e.g. HLA-A3, B27 and B57(7-9)), and Class II antigens (e.g. HLA-DR1101, HLA-DQ0301(10)) with clearance, the temporal association of HCV specific IFN- γ secreting T-cells with

resolution of infection(11), and the generation of polyfunctional, durable CD4+ and CD8+ T-cell subsets directed against multiple HCV antigens in spontaneous control(5, 12). Additionally, the depletion of CD4+ and CD8+ T-cell subsets in chimpanzees is associated with viral persistence following challenge(13, 14), whilst secondary exposure to HCV in intravenous drug users (IVDU) is associated with the generation of robust T-cell immunity(15) which correlates with protection from chronic infection upon subsequent exposure to HCV. Together these data suggest that an HCV T-cell vaccine could prevent persistent HCV infection.

Although broadly neutralising antibodies have been identified(16) and may contribute to viral control in both natural infection(15, 17) and in chimpanzee challenge models after vaccination with HCV envelope proteins(18), it is not yet possible to generate these in most people by vaccination(19). In contrast the development of a potent T-cell vaccine is a practical and attainable goal. Proof-of-principle that a prophylactic T-cell vaccine for HCV may be an effective strategy was first obtained in a chimpanzee model. High levels of anti-HCV specific T-cell immunity were induced in animals vaccinated with adenoviral (Ad) and DNA vectors encoding the non-structural (NS) HCV proteins(20); after heterologous viral challenge 4/5 vaccinated animals developed low-level viraemia and minimal hepatitis, followed by accelerated viral clearance.

In order to overcome the issue of pre-existing anti-Ad immunity in humans, that may limit vaccine efficacy(21), we developed a large panel of replication-defective chimpanzee and human Adenoviruses found at low sero-prevalence that could be used as vaccine vectors(22, 23). We trialled the first HCV prophylactic T-cell vaccine using heterologous Ad vectors derived from human (Ad6) and chimpanzees (ChAd3) encoding the HCV NS3-NS5B polyprotein, with a genetically-inactivated NS5B polymerase(24) in healthy volunteers. The magnitude and breadth of polyfunctional HCV specific T-cells induced after a single priming vaccination with either vector was the most potent described to date in human studies(25). However, there were two limitations; firstly, heterologous Ad boosting failed to increase T-cell responses to the level observed after priming. Subsequent analysis showed that this was most likely due to the induction of cross-reactive anti-Ad antibodies. This was unexpected since heterologous Ad vaccination using the same vectors in macaques had generated substantial responses after boosting, suggesting that the vectors were serologically distinct. Secondly, CD8+ T-cells were the dominant subset induced by vaccination, whereas natural history, genetic and T-cell depletion studies show that both CD4+ and CD8+ T-cell subsets are critical for viral control(5, 11-15, 26-29).

In this study, we have overcome these limitations and developed an HCV prophylactic T-cell vaccination strategy based on heterologous viral vectors (ChAd3 and Modified Vaccinia Ankara; MVA) that is highly immunogenic after both priming and boosting vaccination, inducing both CD4+ and CD8+ T-cells targeting multiple HCV antigenic targets, and associated with an acceptable safety profile.

We elected to use ChAd3 rather than Ad6 since the seroprevalence of ChAd3 is markedly lower than Ad6 (3% vs. 28%), and in a heterologous Ad vectored vaccine regime, boosting following ChAd3 prime was optimal(25). Additionally, we have performed a comprehensive

characterisation of vaccine-induced T-cell phenotype and functionality using both traditional cytometry and (for the first time in a clinical trial) Cytometry by time-of-flight (CyTOF), revealing that this vaccine induced a functional T-cell memory profile.

Results

Vaccination with ChAd3-NSmut and MVA-NSmut is well tolerated

Vaccines were administered intramuscularly, and the volunteer group protocols are described in Table S1.

The majority of local and systemic adverse events (AEs) were mild or moderate (92%) and resolved within 48 hours. Systemic AEs were more common following MVA-NSmut, (mean number/volunteer; 4.1 MVA-NSmut vs. 1.9 ChAd3-NSmut $p=0.032$). The proportion of volunteers experiencing one or more moderate/severe AE was not significantly different between the two vaccines ($p = 0.689$). Severe adverse events were observed in 5 volunteers post-MVA-NSmut and 2 volunteers post-ChAd3-NSmut (local pain/swelling, fatigue, migraine and feverishness) but these resolved within 24-48 hours. Overall, both ChAd3-NSmut and MVA-NSmut were very well tolerated with no serious adverse reactions (fig. S1).

MVA-NSmut optimally and specifically boosts HCV specific T-cell responses after ChAd3-NSmut priming without the induction of Tregs

We previously showed that boosting with heterologous Ad6-NSmut did not enhance anti-HCV immune responses above magnitudes observed with ChAd3-NSmut prime vaccination (25). We therefore evaluated the immunogenicity of a heterologous MVA-NSmut boost (2×10^8 pfu) 8 weeks post ChAd3-NSmut (2.5×10^{10} vp) prime in 9 healthy volunteers (Arm A2, table S1) using IFN- γ enzyme linked immunospot (ELISpot). All volunteers responded to ChAd3-NSmut prime, peaking 2-4 weeks after vaccination (median 1140, range 87-4427 spot forming cells [SFC]/ 10^6 PBMC; fig. 1a). HCV specific T-cell responses were significantly enhanced by MVA-NSmut boost in all volunteers, peaking 1-week post vaccination (median 2355, range 1490-6117, SFC/ 10^6 PBMC; peak post ChAd3-NSmut prime vs. peak post MVA-NSmut boost $p=0.0039$; fig. 1a,b). Furthermore, in comparison to heterologous Ad vaccination, Ch3Ad-NSmut/MVA-NSmut prime/boost generated responses that were more sustained over time, and significantly greater at the end of the study to both the HCV NS region overall, (fig. 1b,c; median 443, range 138-1783 vs. median 98, range 10-1092 SFC/ 10^6 PBMC $p=0.0109$) and to all 6 individual peptide pools covering HCV NS (fig 1d). The peak response post MVA-NSmut boost correlated significantly with the durability of the T-cell response (Trial week 9 [TW9] vs. TW34; Linear regression $R^2=0.784$, $P=0.0034$; fig. 1e) and between TW8 (pre-MVA-NS boost vaccination ($R^2=0.68$ $p=0.0429$)). T-cell responses remained detectable by IFN- γ ELISpot in 4/5 patients tested at weeks 70-73 (median 302, range 10-732 SFC/ 10^6 PBMC; fig. 1a).

No HCV specific T-cell response was detected in the 4 volunteers vaccinated with 2×10^8 pfu MVA-NSmut prime alone (Arm A1; table S3).

To assess a possible non-specific ‘bystander’ expansion of non-HCV Ag-specific T-cells after vaccination, we monitored the T-cell response to HLA class I Flu, EBV and CMV epitopes, and to CMV lysate; no change in the magnitude of the responses to these antigens was observed (fig S2). The induction of regulatory T-cells was also assessed in 5 volunteers pre-vaccination, at the peak of the T-cell response to ChAd3-NSmut prime and MVA-NS boost vaccination (TW2-4 and TW9 respectively), and long-term (TW47-72) after vaccination. We found no significant change in the magnitude of Treg subsets, and levels of Tregs in vaccinated volunteers were comparable to those seen in PBMCs from 8 healthy unvaccinated volunteers (fig S3).

MVA-NSmut boost increases the breadth of ChAd3-NSmut primed T-cell responses

We compared the breadth of T-cell responses induced by ChAd3-NSmut prime/MVA-NSmut boost to that induced by heterologous Ad boosting, using peptides corresponding to the entire immunogen in 6 pools and defined further using 8-11 peptides in mini-pools (table 1). At the peak response following MVA-NSmut boost most individuals responded to all 6 peptide pools (range 4-6; fig. 2a,b), and the breadth was significantly higher than that observed after ChAd3NS-mut prime or after heterologous Ad boost (fig. 2a,c; $p=0.0156$ and $p=0.0010$ respectively). The breadth of response was also significantly greater at the end of the study post MVA vaccination when compared to heterologous Ad boost vaccination ($p=0.0355$; fig. 2a). Further dissection of responses to minipool level revealed that the number of HCV epitopes targeted after MVA boost was as high as 31 in a single individual (subject 310; table 1); because the minipool analysis was performed at TW12, after contraction of the T-cell response, the true number of epitopes targeted in each subject may be underestimated. Although all peptide pools were targeted in patients from diverse HLA backgrounds, responses to NS3 dominated after both ChAd3 prime and MVA boost (Friedman Anova $p=0.0033$; fig. 2b,c). MVA boost increased the magnitude, but did not affect the overall hierarchy of HCV antigen recognition (fig. 2c). Responses to two epitopes located in NS3h restricted by HLA-A1 and HLA-A2 (marked bold in table 1) were particularly prevalent and selected for subsequent pentamer analysis.

All individuals showed a major increase in T-cell response with MVA boost compared to Ad prime but three individuals showed a particularly strong response to MVA boost vaccination (fig. 1a); however, there were no known differences in the 3 “super-responders” when compared to the other volunteers: In particular no one HLA type was over-represented in these 3 volunteers and there was no evidence that these volunteers had prior exposure to HCV. After removing the “super-responders” from the analysis the difference between Ad6-NSmut and MVA-NSmut boost vaccination remained statistically significant at multiple time points, including the end of the study TW34/36 ($p=0.0287$).

Cross reactivity of T-cell responses after MVA-NSmut boosting vaccination

We determined the capacity of the T-cells induced by ChAd3-NSmut/MVA-NSmut encoding the genotype-1b immunogen to target other globally prevalent HCV subtypes using peptides derived from genotypes 1a, 3a, and 4a sequences in IFN- γ ELISpot assays. Although subtypes 1a, 3a and 4a diverge significantly from genotype 1b at the amino-acid level (86%, 77%, and 78% sequence homology respectively) responses to these subtypes

were generated, albeit at a lower magnitude (fig. 2d). Responses to genotype 1a were approximately 60% and genotype 3a/4a 30% of those generated against genotype 1b, whilst breadth is maintained (fig. S4). We observed a direct correlation between the response to the genotype-1b immunogen and subtypes 1a, and 3a but not to 4a (1b vs. 1a: $R^2 = 0.974$, $p = 0.0018$, 1b vs. 3a: $R^2 = 0.975$, $p = 0.0017$, 1b vs. 4a $R^2 = 0.045$, $p = 0.734$).

MVA-NSmut boost induces polyfunctional CD4 and CD8+ T-cell subsets

Next we assessed the relative contribution and functionality of CD4+ and CD8+ T-cell subsets to the total response. We found that MVA-NSmut boosting vaccination induced higher numbers of both T-cell subsets compared to those seen post ChAd3-NSmut prime, and also in comparison to heterologous Ad6-NSmut boost (fig. 3). Using ICS and Spice analysis, we showed that vaccine induced HCV-specific CD4+ and CD8+ T-cells were polyfunctional with an equal proportion of CD4+ T-cells producing one (IL-2 or IFN- γ), two (IL-2 and IFN- γ or IFN- γ and TNF α) or three (IL-2, IFN- γ and TNF α) cytokines, whilst CD8+ T-cells predominantly produced IFN- γ early after vaccination (TW4/TW9) and produced IFN- γ in conjunction with TNF α or TNF α and IL-2 10-14 weeks post boost (fig. S5). The polyfunctionality of CD4+ and CD8+ T-cells increased after MVA vaccination peaking at weeks 18 and 22 respectively (fig. S5). We also assessed the per-cell production of cytokine in polyfunctional compared to single cytokine-producing T-cells (fig. S6). The geometric mean fluorescent intensity (GeoMFI) of each cytokine (with the exception of IL-2 production by CD8+ cells) was significantly higher in CD4+ and CD8+ T-cells that produced three vs. one cytokine. The biggest differences were seen in per-cell production of IFN- γ (median GeoMFI of triple- vs. single-producing CD4+ T-cells was 4,131 vs. 1322 respectively $p < 0.0001$; and 10,329 vs. 1,543 for CD8+ T-cells respectively, $p < 0.0001$). Polyfunctional CD4+ and CD8+ T-cells were readily detectable by ICS 74wks after prime vaccination (fig. 3b).

The proliferative capacity of PBMC was assessed in [^3H]thymidine incorporation assays. HCV recombinant protein antigens were used in this assay, which detects predominantly CD4+ T-cell proliferation. Strong proliferative responses to multiple HCV antigens could be detected 6 weeks after MVA-NSmut boost, and these increased further when assessed 26 weeks after MVA-NSmut boost (Wilcoxon $p = 0.0391$ NS3; fig. 3d), consistent with the generation of a population of memory T-cells capable of rapid proliferation on re-exposure to antigen. Proliferative responses after MVA-NSmut boost were significantly greater than those seen after heterologous Ad boost (fig. 3d).

Detailed characterisation of vaccine-induced CD8 + T-cells using HLA-class I multimers

Following fine mapping of vaccine-induced T-cell responses (table 1), we used HLA-class I pentamers (HLA-A*0201 HCV NS3₁₄₀₆₋₁₄₁₅ KLSALGINAV; HLA-A*0101 HCV NS3₁₄₃₅₋₁₄₄₃ ATDALMTGY) to track the characteristics of vaccine-induced T-cells over time (fig. 4a-b; example FACS plots in fig. S7). After boosting with MVA-NSmut, HCV specific T-cells were highly activated, expressing CD38 in 80-100% pentamer+ cells, with approximately 60% co-expressing HLA-DR (fig. 4c). In contrast, after heterologous Ad boosting 25% expressed CD38 with minimal HLA-DR co-expression. PD-1 expression (a molecule that has been associated with both T-cell activation and exhaustion) was also high

post MVA-NSmut, declining over the duration of the study (fig. 4c). HCV specific T-cells post MVA-NSmut also expressed Granzyme A, and Granzyme B, and variable levels of perforin (fig. 4d).

After ChAd3-NSmut priming vaccination, a mixed pool of memory populations were detected (central memory T-cells [Tcm; CD45RA⁻ CCR7⁺]; effector memory T-cells [Tem; CD45RA⁻CCR7⁻]; naive, or naive-like memory [CD45RA⁺ CCR7⁺]; “terminally differentiated” effector memory T-cells [Temra; CD45RA⁺ CCR7⁻]) that remained after heterologous Ad boosting. In contrast, the large expansion of HCV specific T-cells after MVA-NSmut boost was dominated by CD45RA-populations. Importantly, the long-term memory population at the end of the study after heterologous Ad vaccination was predominantly lymph-node homing (CCR7⁺) T-cells and that had re-expressed CD45RA; in contrast after Ad/MVA regime the dominant population were peripheral organ-homing (CCR7⁻) Tem with low expression of CD45RA (fig. 5).

Analysis of T-cell functionality using CyTOF technology

Single-cell mass cytometry (CyTOF) was used to produce an in-depth analysis of vaccine induced HCV-specific T-cells in two volunteers (319 and 322), at multiple time points. Antibodies labelled with heavy metal isotopes (n=35; table S2) that bind to surface and intracellular proteins allowed T-cell phenotypes to be quantified. HCV-specific T-cells were observed using metal-labelled peptide-MHC tetramers (Gating strategy for CyTOF shown in fig. S8). Validating this approach, the expression of surface and intracellular markers measured by CyTOF correlated with those previously assessed using fluorescent cell analysis (FACS; Spearman's rank $r = 0.8449$ $p < 0.0001$, fig. S9).

We assessed the overall potential for cytokine production of HCV-specific CD8⁺ T-cells after a 3hr stimulation with PMA/Ionomycin so that the cytokine production by HCV-specific T-cells could be compared to that of bulk CD8⁺ T-cells and avoided the rapid down-regulation of TCR and subsequent lack of tetramer staining that accompanies peptide stimulation. Newell et al (30) have previously shown that PMA/Ionomycin stimulation induced comparable cytokine production by T-cells to CD3/CD28 bead activation and that it allowed accurate multimer staining.

In keeping with the ICS data by FACS (fig. S5) we showed a progressive increase in HCV specific CD8⁺ T-cells producing multiple cytokines over time (a feature not seen in the bulk CD8⁺ population) with approximately 80% of cells having 3 functions by trial week 22 (14 weeks post MVA-NSmut; fig. S10). Evidence of a hierarchy of cytokine production by vaccine-induced HCV-specific T-cells was observed, with single cytokine-producing CD8⁺ T-cells making Mip-1 β , dual cytokine-producing T-cells making Mip-1 β and IFN- γ or TNF α , whilst GM-CSF and IL-2 were only produced in combination with Mip-1 β , IFN- γ , and TNF α by the most polyfunctional T-cells (fig. S10).

To further analyse the CyTOF data we employed principal component analysis (PCA). The PCA was loaded with expression data from patient 319 at TW22, as this patient had the largest populations of ‘memory’ CD8⁺ T-cells and a relatively large HCV-specific pentamer cloud (observed by FACS). Although this analysis is unsupervised, the apparent meaning of

each component can be deduced based on previously-defined CD8⁺ T-cell subsets by looking at the markers that most influence the PCs (PC1: naïve vs. memory, PC2: effector function, PC3: T-cell differentiation status; fig. 6a-c). The first three PCs accounted for >50% of the variation within the dataset (fig. 6d) and, therefore, these alone were plotted in PyMol (3D-PCA) to visualise CD8⁺ T-cell complexity. In theory, CD8⁺ T-cells could occupy any space within these plots, however, CD8⁺ T-cells clustered in defined, continuous regions, resulting in an “L-shaped” plot along the PC 1-3 axis; fig. 7. This pattern was observed in both individuals at all time points and in the two vaccine-naïve control patients.

We identified the location of classical and viral specific T-cell subsets on this continuum before defining the location of the vaccine-induced HCV-specific T-cells. Analysis of the relative expression of single markers (e.g. IFN- γ , CD57, CD28, CD45RA and CD27) showed that classical T-cell subsets cluster in discrete niches (fig. 7a and fig. S11). For example, the niche occupied by the naïve T-cell population is easily identified by its high expression of CD45RA, CD27, CD28, and low expression of IFN- γ ; whereas, the majority of non-naïve (memory) T-cells are positioned within an ‘L-Shaped arm’ that extends from the naïve population (fig. 7a). The memory populations were further dissected through the analysis of CD45RA and CCR7, which showed that Tem, Tcm and Temra occupied niches along the L-shaped arm (fig. S11). HCV, Influenza and CMV-specific Tetramer⁺ T-cells were superimposed on the 3D-PCA plot of bulk CD8, showing they also cluster in tightly restricted niches (fig. 7b).

After ChAd3-NSmut prime (TW2) vaccination HCV-specific T-cells appear to occupy a niche close to the naïve population, in the area occupied by Tcm (CD45RA-CCR7⁺) and FLU-specific T-cells (fig. 7b). After MVA-NSmut boost vaccination (TW9) the cells appear more heterogeneous and occupy a broader area across the continuum, with the majority of HCV-specific T-cells matching the position of Tem and CMV⁺ T-cells in the 3D-PCA plots (fig. 7b). At the latest time point studied, TW22, the majority of HCV-specific T-cells sit in a niche at the end of the ‘L-shaped arm’, in a similar location to Temra cells (fig. 7b).

Discussion

We describe the development of a highly immunogenic T-cell vaccine for HCV, using replication-defective chimpanzee Ad and MVA encoding the HCV NS proteins in a prime boost strategy. This approach generates very high numbers of both CD4⁺ and CD8⁺ T-cells, targeting multiple HCV antigens irrespective of host HLA background. Using established technologies and single-cell mass spectrometry (CyTOF), we show that T-cells induced by vaccination are polyfunctional, that functionality increases over time, and that heterologous prime/boost with ChAd3 and MVA induced T-cells with phenotypic and functional profiles distinct from those elicited by heterologous Ad vaccination. Furthermore, the strategy is simple, safe and well tolerated in this phase-I human study.

Whilst the correlates of protection in HCV are not precisely defined, studies of T-cell immunity in natural infection suggest that a number of key parameters will be required. These include the targeting of multiple HCV antigens(12), the generation of CD4⁺ and

CD8⁺ T-cell subsets(11, 12, 14, 15, 26, 28, 29), the maintenance of a memory pool over time with the capacity to proliferate(31), and a population of circulating T-cells with immediate effector function(26, 27). A critical threshold for the magnitude of the T-cell response required has not been established though it is likely that in the context of a prophylactic vaccine “more is better”. The T-cell vaccine regimen described here meets each of these criteria.

The magnitude of the HCV-specific T-cell response generated by heterologous Ad-NSmut/MVA-NSmut vaccination is unprecedented. Priming with ChAd3-NSmut typically induced more than 1000 SFC/10⁶ PBMC, whilst boosting with MVA-NSmut typically doubles this, with responses up to 7000 SFC/10⁶ PBMC in some volunteers. The beneficial effect of MVA boosting is sustained overtime, with a clear elevation in the T-cell “set point” and the maintenance of a sustained memory pool long term. Furthermore, all vaccinees respond to multiple HCV epitopes spanning the NS protein. The association of T-cell breath with spontaneous viral resolution is controversial; some studies suggest that a T-cell response against multiple NS antigens as measured by IFN γ ELISpot is a predictor of viral clearance(5) whilst others have failed to demonstrate a clear association(32). Nevertheless, in the context of a prophylactic HCV vaccine the generation of a broad antiviral response is likely to be important since HCV exists as quasispecies within an infected host and as distinct strains between individuals; targeting multiple HCV antigens increases the likelihood of vaccine-induced T-cells recognising incoming viral strains and escape variants.

In this study we could show that ChAd3/MVA vaccination induced T-cell responses against all six NS antigenic pools in most individuals, and further mapping revealed as many as 31 different epitopes in a single vaccinee.

The ChAd3/MVA vaccination regimen is a significant improvement on the ChAd3/Ad6 regimen previously tested (25); a higher magnitude of T-cell response is seen at all time-points post boost vaccination, and both the breadth and proliferative capacity of the T-cell response are significantly enhanced when measured immediately or long-term after boost vaccination. The T-cells induced by MVA boost vaccination have comparable cytolytic potential and polyfunctionality to those induced by heterologous Ad/Ad vaccination but a distinct combination of T-cell memory phenotypes is induced.

Since HCV exists as distinct genotypes that are broadly segregated geographically, we assessed the capacity of T-cells generated by MVA boost encoding a subtype-1b immunogen to target genotypes 1a, 3a and 4a. Although we have previously shown that T-cell targets in genotype-1 and 3 infection are distinct in the setting of natural infection (33), we find that in the context of a highly immunogenic vaccine, cross-reactive T-cell responses between heterologous viral genotypes are readily generated but at a reduced magnitude. Whether these responses are sufficient to provide protection will require efficacy studies in mixed genotype populations.

Little is known about the ability of potent virally vectored vaccines to induce regulatory T-cell subsets, which may influence vaccine efficacy. The induction of Tregs has been associated with persistent infection in humans (34), and with repeated HCV antigen

exposure in chimpanzees (35). Treg expansion in these studies may reflect priming of T-cells in the tolerogenic liver environment, since we found no induction of Tregs during vaccination with viral vectors encoding HCV antigens in the periphery.

CD4⁺ T-cell responses are known to play a central role in the generation of effective CD8⁺ T-cell immunity(36) and have been reproducibly associated with HCV viral control in both natural infection(28, 29) and in chimpanzee challenge studies(14). Whilst heterologous boosting with MVA-NSmut markedly increased the magnitude of the CD8⁺ T-cell responses compared to heterologous Ad vaccination, the increase in the CD4⁺ T-cell response, producing IL-2, TNF α , and IFN- γ was particularly striking. Furthermore, we show that ability of this important T-cell subset to secrete multiple cytokines is enhanced, and the capacity of CD4⁺ T-cells to proliferate increases over time after MVA-NSmut vaccination.

To increase the resolution of the functional and phenotypic assessment of vaccine induced T-cells we used single cell mass spectrometry (CyTOF). We show that there is a progressive increase in CD8 T-cell polyfunctionality following MVA vaccination, and identified a clear hierarchy of cytokine production (MIP-1- β >IFN- γ >IL-2). Evidence of a hierarchy in cytokine production has been previously described whereby MIP-1- β and IFN- γ are most readily released by T-cells after limited stimulation whereas IL-2 production is only triggered when a T-cell has been exposed to high levels of antigen and co-stimulation(30, 37, 38).

Next, using both CyTOF and conventional Flow Cytometry, we characterised in detail the phenotype of HCV specific T-cells following Ad and MVA vaccination. Significant differences might be expected since the vector for immunogen delivery exerts a profound influence on the type of T-cell response elicited, due to differences in innate signalling pathways and the persistence and quantity of antigen expressed(39, 40). Low levels of transcriptionally active Ad have been shown to persist long-term in both mice(41, 42) and primates(42), whereas transgene expression by MVA becomes undetectable within a few days (43).

We find that the expression of markers of activation and cytolytic capacity are significantly greater following MVA boost. In addition, distinct combinations of T-cell memory phenotypes are seen; following ChAd3 vaccination CD45RA is strongly down regulated inducing dominant populations of Tcm and Tem cells. After heterologous Ad boost the response is dominated by T-cells with a 'naïve' like phenotype (CD45RA+CCR7+), despite the fact that these are functional antigen-experienced T-cells. In contrast, after MVA-NSmut boost there is a marked expansion of Tem cells that increase further over time so that by the end of the study ~50% of cells are Tem, ~30% display a Temra phenotype and ~10-15% appear to be Tcm or "naïve-like" in phenotype. Through 3D-PCA analysis of the CyTOF data, we confirmed the previous observation that antigen experienced cells exists along a continuum that extends from naïve towards memory populations, associated with a progressive loss of markers associated with naïve/early differentiated cells (CD28/CD27/CCR7) and a gain in expression of markers associated with senescence (CD57, CD45RA) (30). We show that vaccine-induced HCV-specific T-cells cluster in restricted niches; after Ad priming HCV-specific T-cells cluster near the area occupied by influenza-specific and

Tcm CD8+ T-cells. In contrast after MVA boost these become more heterogeneous, occupying areas further along the differentiation pathway with a phenotype that is more typical of a CMV-specific T-cell population.

This evolution of CD8+ T-cell memory is striking, as a contraction towards a Tcm phenotype might have been expected. Overall, the phenotype of T-cells after Ad/MVA vaccination are remarkably similar to those induced by the highly efficacious yellow fever and smallpox (Dryvax) vaccines (i.e. Temra/Tem phenotype and PD-1+ expression), which are associated with life long protection(44). Furthermore, Tem and Temra cell subsets have been associated with protection against HIV(45) and Influenza(46) in natural history studies, and against SIV(47) and Malaria(48)) in vaccine studies. It is plausible that low-level persistence of Ad-derived transcripts contributes to the moulding and maintenance of long-lived Tem populations; recent data from studies of recombinant Ad vaccines in mice reveals long-term evolution of sustained Tem populations, with a close resemblance to expanded CD8+ T-cell memory induced after CMV infection, and akin to those described here(41, 49).

Whilst the diversity of the HCV genome represents a major challenge to vaccine development, a proportion of people infected with HCV are able to eradicate virus spontaneously and effective T-cell immunity appears to play a crucial role in this. Overall, we have generated a potent T-cell vaccine that we believe may recapitulate and accelerate these events *in vivo* to prevent the development of chronic disease, thus paving the way for the first human efficacy studies. Suitable cohorts of IVDU populations have now been identified(50, 51) and the first efficacy study of ChAd3NSmut/MVA-NSmut in IVDUs has recently started in the USA (NCT01436357). This study will enable the assessment of vaccine immunogenicity, efficacy and safety in a larger cohort of volunteers with a broad range of HLA-types, exposed to different viral sub-types.

Materials and Methods

Study design

The two primary end points in this study are safety and immunogenicity. The ChAd3-NSmut/MVA-NSmut vaccine study (HCV003) is registered in the [ClinicalTrials.gov](https://clinicaltrials.gov/) database (ID: NCT01296451). All volunteers gave written informed consent prior to enrolment and the studies were conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice (GCP). Volunteers were recruited at the CCVTM (Centre for Clinical Vaccinology and Tropical Medicine), Churchill Hospital, Oxford. Vaccines were administered intramuscularly (ChAd3-NSmut dose, 2.5×10^{10} viral particles [vp]; MVA-NSmut dose, 2×10^8 Plaque forming units [pfu]). Volunteers were observed for 1-3 hours following vaccination. A dose escalation for ChAd3-NSmut is described in (25). The MVA dose was selected based on the use of MVA vectors in human studies (52, 53). Volunteers systematically documented all symptoms and recorded a daily oral temperature. Solicited and unsolicited local and systemic adverse events were collected in diary cards and recorded in case report forms. A total of 24 volunteers were assessed for eligibility and 15 volunteers were enrolled in one of two arms. Four individuals were enrolled into Arm A1 and vaccinated with MVA-NSmut alone. Only after vaccine safety

had been established in this arm were volunteers enrolled into arm A2. A total of eleven volunteers were enrolled into arm A2. One individual withdrew consent after receiving ChAd3-NSmut but before receiving MVA-NSmut. Hence 10 volunteers completed the vaccine schedule in arm A2. One of these 10 individuals received a lower dose of ChAd3-NSmut as the required volume could not be extracted from the vaccine vial. Safety data from all 15 volunteers is reported, however immunogenicity data is reported for all the A1 volunteers and the 9 volunteers in arm A2 who completed the vaccination schedule with the doses stated above. An additional two volunteers vaccinated with the arm A2 schedule subsequently included for assessment of Treg subsets only (fig S3). Both exposure to HCV (defined as HCV Ab and HCV RNA positivity) and recent intravenous drug use were study exclusion criteria. HCV Abs, quantitative HCV RNA, and T-cell response to HCV antigens were all undetectable at baseline. There were no signs or symptoms of viral hepatitis or an increase in liver enzymes during the study.

Comparisons are made with the previously published ChAd3-NSmut/Ad6-NSmut vaccine study (HCV001; group 10 ChAd3-NSmut dose 2.5×10^{10} vp; group 11; ChAd3-NSmut dose 7.5×10^{10} vp; doses were combined for analysis since we have previously shown that there is no dose effect above 2.5×10^{10} vp) registered as (ID: NCT01070407; n=9; safety data and immunology previously published(25)). Study Groups and vaccination regimes in study HCV001 and HCV003 are detailed in table S1.

Adenoviral constructs

The Ad6 and ChAd3 vectors encoding the NS3-5B region (1985 amino acids) of genotype 1b BK strain (based on sequence accession number M58335) have been described previously(20, 22) and were manufactured at the Clinical BioManufacturing Facility (CBF) Oxford University.

Generation of the MVA-NS

The NSmut expression cassette was subcloned into the MVA shuttle vector pMVA-GFP-TD flanked by TKL (thymidine kinase gene left region) and TKR (thymidine kinase gene right region) generating the transfer vector pMVA-GFP-TD-NSmut. pMVA-GFP-TD-NSmut, drives the antigen expression using the vaccinia P7.5 early/late promoter, and expression of Green Fluorescent Protein (GFP) using the fowlpox late promoter, FP4b. The production of the recombinant MVA-NSmut virus was based on *in vivo* recombination between the MVA-Red genome and homologous sequence (TKL and TKR) within the transfer vector pMVA-GFP-TD-NSmut. PCR was performed to check for the presence of NS transgene and absence of wt-MVA and MVA-Red virus.

Peptides and antigens

A set of 494 peptides, 15 amino-acids (aa) in length, overlapping by 11 aa and spanning the open-reading frame from NS3 to NS5B (1985aa) of HCV genotype 1b strain BK (matching the vaccine immunogen) were obtained from BEI resources. Peptides were initially dissolved in dimethyl sulfoxide (DMSO) and arranged into six pools labelled F to M and corresponding respectively to NS3p, NS3h, NS4, NS5A, NS5B I, NS5B II (mean 82, range 73-112 peptides/pool). Pools were used with each single peptide at a final concentration of

3µg/ml or 1µg/ml in ELISpot and Intracellular Cytokine Staining (ICS) assays respectively. For cross-reactivity experiments, similar peptide pools derived from HCV genotype 1a (H77 strain), genotype 3a (Genbank accession D28917), and genotype 4a (ED43; EMBL accession Y11604) were prepared identically. Optimal length peptides were purchased from Proimmune Ltd (Oxford, UK).

ELISpot assays

Ex-vivo IFN-γ ELISpot assays were performed as described previously on freshly isolated peripheral blood mononuclear cells (PBMC) plated in triplicate at 2×10^5 PBMC per well(25). A robust positive cut-off was previously calculated from 74 healthy HCV seronegative volunteers. For a positive response; (i) the mean of antigen wells was determined to be greater than 48 SFC/ 10^6 PBMC (mean + 3 SD) and (ii) to exceed 3x background. Background wells (medium only, cells + DMSO) were typically 0-4 spots. Internal positive controls included Concanavalin A, FEC (mixed HLA class-I restricted peptides from Flu, EBV and CMV) and a CMV lysate. Total NS response was calculated by summing responses to all positive pools (NS3p-NS5B II) and corrected for background.

Cells were counted using a Guava Personal Cell Analysis system (Merck Millipore) as previously described(25).

Proliferation assays

Ex-vivo proliferation assays were performed on freshly isolated PBMC plated in triplicate at 2×10^5 PBMC per well using conventional [3 H]thymidine incorporation methods and HCV proteins (1µg/ml) as described previously(25). Data are displayed as SI (Stimulation Index; fold change above background). A positive response is defined as SI ≥ 3 .

Intracellular cytokine staining (ICS)

ICS was performed as described previously(25) on fresh PBMC or thawed PBMC after 3hrs resting at room temperature. Briefly, PBMC were stimulated using peptides in pool combinations (F+G+H=NS3-4, I+L+M=NS5A-B, 1µg/ml), or with individual pools, peptides (15mers and optimal 8mers, 1µg/ml), unstimulated (controlled for DMSO), or PMA/Ionomycin (50 and 500 ng/ml respectively). After overnight stimulation (Brefeldin A was added after 1hr at 10 µg/ml), cells were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen; used in all FACS assays), fixed (1% paraformaldehyde), permeabilised (ebiosciences 10x perm buffer) and stained with the following antibodies: CD3-PO, CD4-Qdot 605, CD8-PerCP Cy5.5, IFN-γ-AlexaFluor700, IL-2-APC, TNFα-PE-Cy7 (table S2).

For analysis of peptide-specific function, PBMC were stimulated with peptide at 1µg/ml or control DMSO or PMA/Ionomycin. ICS was then performed as described above, stained with the following antibodies: CD3-PO, CD4-Qdot 605, CD8-PB, IFN-γ-AlexaFluor700, IL-2-APC, TNFα-PE-Cy7, MIP-1β-PE (table S2).

Flow cytometry was performed using a BD LSRII and analysis by FlowJo (TreeStar). Analysis of polyfunctionality was performed using Pestle, and SPICE version 5.3, downloaded from <http://exon.niaid.nih.gov>(54). All ICS data are corrected for background.

Pentamer and Treg staining, short term cell lines and flow cytometry

PE-labelled pentamers loaded with HCV NS3₁₄₀₆₋₁₄₁₅ (KLSALGINAV; HLA-A*0201), or HCV NS3₁₄₃₅₋₁₄₄₃ (ATDALMTGY, HLA-A*0101) were obtained from ProImmune (table S2 for list of antibodies and fluorochromes costained with pentamers). The specificity of pentamers was tested on HLA-matched pre-vaccination samples from healthy volunteers (25).

Pentamers were centrifuged at 4°C (1400g 10mins) and 1µl was taken from the supernatant and used to stain 1-2×10⁶ fresh or thawed PBMC in 50µl PBS. PBMC were then sequentially: stained with fixable NIR LIVE/DEAD stain, fixed (1% paraformaldehyde), permeabilised (for internal stains only; ebiosciences 10x perm buffer), and then stained with surface or internal antibody cocktails separately for 30mins. Fluorescent markers were observed and analysed using a BD LSRII and FlowJo software (TreeStar). CD45RA/CCR7 subsets were analysed using Pestle and SPICE. Fluorescence minus one (FMO) samples were performed using a CMV pentamer on CMV+ PBMC and fixed gating strategies were used throughout.

For quantification of Treg subsets frozen PBMC were thawed and stained with fixable NIR LIVE/DEAD stain, surface stained with CD3-PO, CD4-Qdot605, CD25-Pecy7, CD8-PB, CD127-APC antibodies, then fixed, permeabilised and stained with FoxP3-Alexa700 according to ebiosciences One-step protocol for intracellular (nuclear) proteins using the Foxp3 / Transcription Factor Staining Buffer Set (Cat: 00-5523-00).

Mass cytometry using CyTOF

T-cell phenotype and function was determined by mass cytometry analysis in volunteers 319 and 322 at TW2/4 (peak T-cell immunogenicity after ChAd3-NSmut vaccination), TW9 (peak T-cell immunogenicity after MVA-NSmut vaccination), and at TW22, performed on frozen PBMC.

Detailed methodology is described elsewhere(30). Briefly, cryopreserved cells were thawed and left overnight at 37°C. Dead cells were then removed by ficoll density separation. For stimulation, cells were cultured for 3 hours with 150ng/ml PMA + 1mM Ionomycin in the presence of brefeldin A, monensin and anti-CD107α/β. Cells were then stained with a cocktail of metal-conjugated surface antibodies (n=35) and tetramers (n=3; table S2). Cells were fixed, left overnight, and then permeabilised and stained with metal-conjugated antibodies for intracellular markers and with DNA interchelators (iridium, DVS Sciences).

Purified antibodies were labelled with heavy metal-preloaded maleimide-coupled MAXPAR chelating polymers via the 'Pre-Load Method v1.1 as previously described(30). Cells were also stained with three fluorochrome labelled flow antibodies (CD161-APC, gamma-delta-FITC, and PD-1-PE) and then stained for anti-PE, anti-APC, and anti-FITC antibodies

conjugated to metals. CD3 was stained using a Qdot antibody, which is composed of cadmium.

Tetramer generation was performed as previously described(55), briefly: HLA-A*0201 MHC molecules folded with UV-cleavable peptides were biotinylated and purified. Peptides were exchanged by UV irradiation of MHC in presence of high concentrations of CMV pp65₄₈₂₋₄₉₀ NLVPMVATV, FLU M1/MP₅₈₋₆₆ GILGFVFTL, HCV NS3₁₄₀₅₋₁₄₁₅ KLSALGINAV. pMHC molecules were then tetramerised and labelled with heavy metal isotopes-coupled to streptavidin. Tetramers were multiplexed by conjugating each tetramer to an exclusive combinations of two heavy metals for each tetramer-peptide specificity (table S2)(56). Data were acquired and analysed on the CyTOF machine as previously described(30). The specificity of tetramers was tested on PBMC from two HLA-matched unvaccinated individuals.

Mass cytometry Data Analysis

Built-in cell-identifying software on the CyTOF creates an FCS file, enabling mass cytometry data to be analysed in a manner similar to standard flow cytometry data by FlowJo software (Treestar, Inc.).

For principal component analysis (PCA) cells were gated on live CD3+CD8+ T-cells (principal component loaded using data from patient 319 at TW22 then applied to the other datasets) and these events were exported to a tab-delimited text file with FlowJo v9.3.2 for further analysis with scripts written in Matlab. FCS files containing these additional parameters were created using a custom algorithm written in Java (text to FCS script generously provided by W. Moore). pdb files were also created using Matlab that could be read by PyMOL software (DeLano Scientific LLC). All Matlab scripts started with transformation of data into logicle biexponential scaling as described(30).

Statistical analysis

Nonparametric tests were used throughout, paired for within-individual comparisons (Wilcoxon) and unpaired for group comparisons (Mann Whitney). For correlations a nonparametric test was used (Spearman). For multiple comparisons a one-way Anova with Bonferroni correction was used. Prism (v6.0 for Mac) was used throughout. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. Only statistically significant results are reported in figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Magnitude of T-cell responses to HCV NS after vaccination

a-d) The total *ex-vivo* IFN- γ ELISpot response to the NS region of HCV is shown over time during the vaccine trial (calculated by summing the responses to positive peptide pools corrected for background; see methods). **a)** The kinetics of the response is shown for the nine volunteers who received ChAd3-NSmut/MVA-NSmut vaccination. 4 volunteers were assessed at an extra time point a year and half after initial vaccination (TW70-73). **b)** The median *ex-vivo* IFN- γ ELISpot response to HCV NS is shown for volunteers receiving ChAd3-NSmut/MVA-NSmut (black; n=9) or ChAd3-NSmut/Ad6-NSmut (grey n=9) vaccine regimens. Arrows indicate vaccinations and trial week indicates weeks since prime vaccination. (ChAd3/MVA TW12 vs ChAd3/Ad6 TW12 p=0.0012; ChAd3/MVA TW9 vs ChAd3/Ad6 TW10 p=0.0009) **c-d)** A comparison of the total *ex-vivo* response to HCV NS at TW34-36 in volunteers receiving ChAd3-NSmut/MVA-NSmut (black) or ChAd3-NSmut/Ad6-NSmut (grey) vaccinations (p = 0.0109) by IFN- γ ELISpot; **c)** magnitude of summed total T-cell response to HCV NS. **d)** magnitude of T-cell response to six peptide pools covering HCV NS. **e)** The magnitude of response to HCV NS at the peak after MVA-NSmut vaccination (TW9) vs. the end of the study (TW34) for volunteers receiving ChAd3-NSmut/MVA-NSmut vaccinations with linear regression (no TW34 data available for volunteer 320).

Figure 2. Breadth, specificity and cross-reactivity of T-cell responses to HCV NS after vaccination

a) The *breadth* of the T-cell response to HCV NS (the number of positive pools, F-M) assessed *ex-vivo* at peak magnitude after ChAd3-NSmut prime vaccination (TW2-4; open circles), after MVA-NSmut boost (TW9; black triangles) or Ad6-NSmut boost (TW12-16; grey triangles), and at the end of the study (EOS; TW34-36) after MVA-NSmut boost (black squares) or Ad6-NSmut boost (grey squares) measured by IFN- γ ELISpot. Bars, median. (ChAd3 prime vs MVA boost $p=0.0156$; Ad6 boost vs MVA boost $p=0.0010$; Ad6 EOS vs MVA EOS $p=0.0355$) **b)** The *magnitude* of the T-cell response to individual peptide pools 1 week after boost (TW9) is shown for volunteers who received ChAd3-NSmut/MVA-NSmut vaccination (IFN- γ ELISpot) **c)** The peak *magnitude* of the T-cell response to each individual pool is shown for volunteers vaccinated with ChAd3-NSmut/MVA-NSmut ($n=9$) after ChAd3-NSmut prime vaccination (TW2-4; open circles) and after MVA-NSmut boost vaccination (TW9; black triangles; IFN- γ ELISpot; ChAd3/Ad6 vs ChAd3/MVA pool F $p=0.0106$, pool G $p=0.0106$). **d)** The *cross-reactivity* of T-cell response: The total magnitude of T-cell responses using peptide pools covering the NS region of HCV genotype 1b

(vaccine strain) compared to (i) genotypes 1a, 3a and (ii) genotypes 4a in volunteers by IFN- γ ELISpot assay at TW9. Bars at median with values shown (SFC/10⁶ PBMC).

Figure 3. Functionality of vaccine-induced CD4+ and CD8+ T-cells

a-b) Example FACS plots showing TNFα/IFN-γ and IL-2/IFN-γ after intracellular cytokine staining are shown for CD4+ and CD8+ T-cells stimulated with NS3-4 or DMSO control, in volunteer 310 one week (**a**) and 62 weeks (**b**) after MVA-NSmut boost vaccination (TW9 and TW70 respectively). **c)** A comparison of cytokine production by T-cells at peak response post vaccination. The percentage of total CD4+ or CD8+ T-cells producing IFN-γ, TNFα, or IL-2 after stimulation with NS3-5 is shown at the peak after ChAd3-NSmut prime vaccination (open circles; TW4, n=16) and at the peak after boost vaccination with either

heterologous Ad6-NSmut (grey triangles; TW12, n=8) or MVA-NSmut (filled circles: TW9, n=9). PBMC were stimulated with pools F+G+H (NS3-4) or I+L+M (NS5) overnight and the percentage of cytokine secreting cells for these two stimulations were summed after background subtraction (DMSO stimulation) to get the total NS response. Values ≤ 0.01 are assigned 0.01. Bars, Median. **d) Proliferative capacity of T-cells after boost vaccination:** The proliferative response to stimulation with HCV proteins, plotted as box and whisker plots (max-min, IQR and median), is shown 6-8 weeks (TW14/16) and 26-28 weeks (TW34/36) after boost vaccination for ChAd3-NSmut/Ad6-NSmut (checkered bars; n=9) and ChAd3-NSmut/MVA-NSmut (solid bars; n=9) groups. Data are expressed as Stimulation index (SI). A dashed line at SI=3 indicate positivity cut off (Data shown in table S4).

Figure 4. Phenotyping of vaccine-induced HCV-specific T-cells

a) Example FACS plots of staining with tetramer A2-HCV-NS3₁₄₀₆₋₁₄₁₅ in volunteers 319 and 322 (vaccinated with ChAd3-NSmut/MVA-NSmut) over the study time course. Gating is on live CD3⁺ cells. Values indicate Percentage of CD8⁺ cells binding pentamer. **b)** *Magnitude of pentamer cloud:* The percentage of CD8⁺ T-cells binding pentamer (HLA-A2-HCV₁₄₀₆₋₁₄₁₅ or HLA-A1-HCV₁₄₃₅₋₁₄₄₃) is shown for individual volunteers over the study time course. **c-d)** *Phenotype of vaccine induced T-cells:* The percentage of the pentamer⁺ cells expressing phenotypic markers CD38, HLA-DR and PD-1 **c)**, or granzyme A,

granzyme B, and perforin **d**), are shown 4 weeks after ChAd3-NSmut prime (open circles; peak prime-PP), after Ad6-NSmut boost (PB = post boost, TW12; EOS = end of study, TW36 grey, n=5) and after MVA-NSmut boost (PB, TW9; EOS, TW34 respectively; black, n=7). Comparisons are made between vaccine regimens and between time points within a single regimen (ChAd3-NSmut/Ad6-NSmut vs ChAd3-NSmut/MVA-NSmut). Only statistically significant differences are shown. All pentamer staining and phenotyping performed *ex vivo* without culture. Example FACS plots shown in fig. S7.

Figure 5. Memory phenotype of vaccine-induced CD8 T-cells

PBMC were costained with pentamers to immunodominant epitopes in NS3 and with antibodies to human CD45RA and CCR7 *ex-vivo*. Data is shown at the following timepoints: Post ChAd3-NSmut prime (TW4, n=8), peak post Ad6-NSmut boost (TW12, n=5), at the end of the study (EOS) after ChAd3-NSmut/Ad6-NSmut (TW36, n=5; EOS ChAd3/Ad6), peak post MVA-NSmut boost (TW9, n=7) and at the end of the study after ChAd3-NSmut/MVA-NSmut (TW22-74, n=7; EOS ChAd3/MVA). **a)** Pie charts show the proportion of the Pentamer⁺ cells which display the Naïve, Tem, Temra, Tcm phenotype at the time points listed above. Pie base, Median. **b)** The bar graph shows the percentage of Pentamer⁺ cells displaying a particular phenotype at the time points listed above. Median and upper quartile are shown.

Figure 6. Principal component analysis (PCA) of T-cell immunity in human vaccinees

The PCA was loaded with the relative expression of the markers listed on the x-axis of the bar graphs above (a-c) for all CD8⁺ T-cells from volunteer 319 at TW22. PCA produces summary variables/principal components that summaries as much variation in the expression of these markers across CD8 T-cells as possible. **a-c)** The three components (PC1, PC2, PC3) summarising the most variation are shown with the weighting coefficients/component loading for each marker. **d)** The percentage of the overall variation in the markers assayed on CD8 T-cells explained by each principal component is plotted individually (bars) and cumulatively (line). The first three components, shown in **a-c)** and used in the 3D-PCA plots in Fig. 7 are shown by filled bars/dots.

Figure 7. 3D Principle Component Analysis of vaccine-induced CD8+ T-cells

The first three principal components were plotted using the protein imaging program PyMOL (PC1 axis in red, PC2 axis in green and PC3 axis in blue). **a)** Bulk CD8 T-cells. Each dot represents a single CD8 T-cell and in the images above the cells are coloured (blue = low, red = high) according to their relative expression within the CD8 population of IFN- γ , CD57, CD45RA, CD28, and CD27. A plot showing all CD8 T-cells as grey dots shows the shape of the CD8 T-cells in space according to the first 3 PCs with the relative locations of Naïve, T_{cm} and Temra populations highlighted by arrows. Data from volunteers 319 and

322. **b)** 3D-PCA of vaccine-induced HCV-specific T-cells: Each pink dot represents a single CD8⁺ T-cell (bulk CD8⁺ T-cells from 319 and 322) and plotted on the same axis are the tetramer⁺ (NS3₁₄₀₆₋₁₄₁₅ HLA-A2) T-cells at TW2 (peak after ChAd3-NSmut prime, green) at TW9 (peak after MVA-NSmut boost, red) and at TW22 (blue). The location of CMV (black) and FLU (purple) specific T-cells, stained using tetramers on PBMC from healthy unvaccinated volunteers (LC037 and LC046), are also shown. The bulk CD8⁺ T-cells are shown in the bottom left image, coloured for their relative expression of IFN- γ with the locations of Naïve, Tcm and Temra populations highlighted by arrows for reference.

Table 1

Specificity of T-cell response to HCV NS after vaccination

The peptide pools that were positive (see methods; >48 SFC/10⁶ PBMC and 3X DMSO by ELISpot assay) at the peak of the T-cell response after ChAd3-NSmut/MVA-NSmut vaccination (TW9) are shown with each volunteer’s HLA-type. Where possible T-cell responses were further mapped to minipool (at TW12), 15mer peptide or optimal peptide. The immunodominant responses to peptides 103 and 95 (Pool G; NS3) in HLA-A1 and HLA-A2 donors respectively are shown in bold and were tracked using MHC class I multimers.

Volunteer	HLA	A	HLA	B	HLA	C	Positive pools	Minipools	Peptide (minipool/peptide number-sequence)	Optimal (HLA Restriction)
304	11	11	39	57	12	6	F	Fb, Fd, Ff, Fh	Fb:11-QSELATCVNGVCWTV/12-ATCVNGVCWTVYHGA	Fb: CVNGVCWTV (A2)
							G	Ge, Gf, Gg, Gh	Gh:153-VTLTHPTIKYIMACM	Gh: TLTHPTIK (A11)
							H	Ha, Hd, Hf	Ha:164-SVVIVGRIILSGRPA; Hf:207-ILAGYGAGVAGALVA	
							I	Ib, Ic, Id, Ii		
							L	Lc, Lf	Lf:400-LVNTWWSKKNPMGFS/401-WSKKNPMGFSYDTR	Lf: KSKKNPMG (B57)
305	11	1	44	52	5	12	M	Me, Md, Mf	Mc: 444-RVYYLTRDPTTPLAR/445-LTRDPTTPLARAAWE	Mc: LTRDPTTPLAR
							F	Ff, Ff, Fh	Ff: 56-QGYKVLNPSVAAT/57-VLVLNPSVAATLFGF	Ff: VLVLNPSVAAT
							G	Gc, Gc, Gg, Gh	Gc: 103-VATDALMTGYTGDFD; Gh: 149-HGPTPLLYRLGAVQN/150-PLLYRLGAVQNEVTL	Gc: ATDALMTGY (A1); Gh: PLLYRLGAVQN
							H	Ha, Hd		
							I	Ii		
308	2	1	8	44	5	7	M	Mf		
							F	Fb	Fb:11-QSELATCVNGVCWTV/12-ATCVNGVCWTVYHGA	Fb: CVNGVCWTV (A2)
							G	Gb, Gc	Gb: 95-LAAKLSGLGINAVAY; Gc: 103-VATDALMTGYTGDFD	Gb: KLSGLGINAV (A2); Gc: ATDALMTGY (A1)
							H	Ih		
							I			
309	11		35	4			L			
							M	Mf		
							F	Fg		
							G	Ga, Gg, Gh	Ga: 83 VTVPHPNIEEVALSN/84-HPNIEEVALSNTGEI; Gh: 153-VTLTHPTIKYIMACM	Ga: HPNIEEVAL (B35); Gh: TLTHPTIKY (A11)
							H	Hd		
310	3	2	35	65	4	8	I			
							M			
							F	Fa, Fb, Fc, Fd, Ff, Fh	Fb:11-QSELATCVNGVCWTV/12-ATCVNGVCWTVYHGA	Fb: CVNGVCWTV (A2)

Volunteer	HLA	A	HLA	B	HLA	C	Positive pools	Minipools	Peptide (minipoolpeptide number-sequence)	Optimal (HLA Restriction)
319							G	Ga, Gb, Gc, Gd, Ge, Gf, Gg, Gh,	Ga: 83-VTVPPHPNIEEVALSN/84-HPNIEEVALSNTGEI; Gb: 95-LAAKLSGLGINAVAY	Gb: KLSGLGINAV (A2)
							H	Ha, Hb, Hd, He, Hf, Hg, Hh	Hf:207-ILAGYGAGVAGALVA	
							I	Ib, Ic, Id, Ie, If, Ig, Ij,		
							L	Lc, Le	Le:391-RVCEKMALYDVVSTL	
							M	Mg		
							F	Fa, Fb, Fd	Fa:10-STATQSFATCNGV; Fb:11-QSFLATCNGVCWTV/12-ATCVNGVCWTVYHGA	Fb: CVNGVCWTV (A2)
							G	Gb, Gc, Ge, Gf	Gb: 95-LAAKLSGLGINAVAY; Gf: 129-LRAYLNTPLGYPVCQD	Ga: HPNIEEVAL (B35); Gb: KLSGLGINAV (A2)
							H	Hf	Hf:207-ILAGYGAGVAGALVA	
							I	Ic, Id	Ic: 264-SRALWRVAEEYVEV; Id: 274-PEFFTEVDGVR LHRY	
							L	Lc, Lf	Lf:400-LVNTWKSKKNPMGFS/401-WKSKKNPMGFSYDTR	Lf: KSKKNPMG (B57)
320							G	Gb, Gc, Gd	Gb: 97-GINAVAYRGLDVSV;Gc: 103-VATDALMTGYTGDFD; Gd: 118-RRGIYRFVTPGERPS	Gc: ATDALMTGY (A1)
							H			
							I	Ic		
							L			
							M			
							F	Fb	Fb:11-QSFLATCNGVCWTV/12-ATCVNGVCWTVYHGA	Fb: CVNGVCWTV (A2)
							G	Gb	Gb: 95-LAAKLSGLGINAVAY	Gb: KLSGLGINAV (A2)
							H	Hh		
							M	Md, Mg	Md: 454-WARMILMTHFFSILL; Mg: 489-IYHLSLRARPRWFML/490-LSRARPRWFMLCLLL	
							F			
322							G	Gc, Gd, Gh	Gc: 103-VATDALMTGYTGDFD; Gd: 109-TQIVDFSLDPTFTTIE/110-DFSLDPTFTTETTV; Gh: 153-VTLTHPTKYIMACM	Gc: ATDALMTGY (A1); Gh: TLTHPTKY (A11)
							H	Hb	Hb: 167-RPAIVPDREFLYQEF	
							I			
							L	Le	Le:388-PARLIVFPDLGVRVC	
							M			
324										