EXPERT OPINION

Ever closer to a prophylactic vaccine for HCV

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Introduction: With 3 – 4 million new infections occurring annually, hepatitis C virus (HCV) is a major global health problem. There is increasing evidence to suggest that HCV will be highly amenable to a vaccine approach, and despite advances in treatment, a vaccine remains the most cost-effective and realistic means to significantly reduce the worldwide mortality and morbidity associated with persistent HCV infection.

Areas covered: In this review we discuss immune responses to HCV during natural infection, and describe how they may inform vaccine design. We introduce the current candidate vaccines for HCV and compare how these fare against the expected requirements of an effective prophylactic HCV vaccine in relation to the breadth, functionality, magnitude and phenotype of the vaccine-induced immune response.

Expert opinion: Although the correlates of immune protection against HCV are not completely defined, we now have vaccine technologies capable of inducing HCV-specific adaptive immune responses to an order of magnitude that are associated with protection during natural infection. The challenge next is to i) establish well-characterised cohorts of people at risk of HCV infection for vaccine efficacy testing and ii) to better understand the correlates of protection in natural history studies. If these can be achieved, a vaccine against HCV appears a realistic goal.

Keywords: functionality, hepatitis C virus, immunity, phenotype, prophylactic, T cells, vaccines, viral vectors

Expert Opin. Biol. Ther. (2013) ***13****(8):1109-1124*

1. Introducing the problem

The prevention of persistent hepatitis C virus (HCV) infection is an area of real unmet clinical need. Of the estimated 3 – 4 million new HCV infections per year, 10 – 20% will go on to develop chronic liver disease and HCV is now the most common indicator for liver transplantation in many countries [1,2]. HCV is able to persist in up to 70% of immune-competent hosts it infects, and this leads to a state of chronic hepatic inflammation, which can progress to fibrosis and cirrhosis of the liver, and ultimately liver failure or hepatocellular carcinoma [1,3].

It was previously thought that an effective vaccine against HCV would be impossible; however, we now know that a significant number of individuals spontaneously clear the virus in the setting of an appropriate immune response, and there is evidence of protective immunological memory against HCV in chimpanzees (*Pan troglodytes*) and humans, where secondary infection is associated with reductions in peak viral titre, duration of viraemia, hepatic inflammation and an increased rate of viral clearance [4-6]. Immunological memory does not appear as effective as is seen with Hepatitis A, B or E, as it is rarely, if ever, sterilising (reviewed in [4,6-8]); however, an attenuated course of infection associated with early viral clearance prevents chronicity and significant liver disease [4,6]. The goal for a vaccine against HCV is unique in that to prevent the majority of disease it need only prevent persistence of the virus rather than prevent infection.

informa
healthcare

10.1517/14712598.2013.791277 © 2013 Informa UK, Ltd. ISSN 1471-2598, e-ISSN 1744-7682

Article highlights.

- There is currently no approved vaccine for HCV, which is a major global health problem, newly infecting ~ 3 – 4 million people annually worldwide.
- HCV persists in up to 70 – 80% of immune-competent hosts it infects, leading to a state of chronic hepatic inflammation that can progress to liver failure or hepatocellular carcinoma.
- The goal for a prophylactic vaccine against HCV is unique in that to prevent disease it need only prevent persistence of the virus, rather than prevent infection.
- Antibody responses to HCV are often strain specific or do not neutralise circulating HCV strains because they target highly variable sequences.
- We now know that a small but significant proportion of humans and NHPs spontaneously clear HCV and immunological memory can provide protection on reinfection. Initial clearance of HCV and protection against persistence in subsequent infections appear to be prominently mediated by cellular immunity.
- Current HCV vaccine approaches include peptide, plasmid DNA, recombinant proteins and vector-based vaccines.
- Virally vectored vaccines are the most promising in terms of T-cell induction – in particular Ad and MVA vectors.
- There is an ever-increasing complexity seen within T lymphocyte populations and phenotype has been linked to functionality and protection; therefore, an understanding of the combination of functions possessed by the T-cell population is needed to effectively assess vaccine efficacy.
- Parameters of interest when assessing a vaccine-induced HCV-specific T-cell population include breadth, cytokine production, cytolytic capacity, magnitude, phenotype and proliferative capacity.
- New technologies capable of analysing multiple T-cell parameters simultaneously may enhance the stratification of T-cell subpopulations by function, allowing identification of better correlates of protection for HCV.
- A major challenge in the next era of HCV vaccinology will be the assessment of vaccine efficacy in well-characterised at-risk populations.

This box summarises key points contained in the article.

Currently, there is no licensed vaccine for HCV and treatment is based on pegylated-interferon-α (IFNα) and the nucleoside analogue ribavirin. This is expensive, relatively toxic, prolonged (24 – 48 weeks) and leads to a sustained virological response (SVR) in only 50 – 60% of patients, depending on the infecting genotype [9]. New directly acting antivirals (DAAs) that target specific HCV proteins are emerging for the treatment of HCV. Two first-generation protease inhibitors boceprevir (Victrelis®, Merck) and telaprevir (Incivek®, Vertex; Incivo®, Johnson & Johnson) have been approved for use in the treatment of genotype 1 HCV, and newer DAAs targeting a wider spread of genotypes and offering increased SVR rates are expected to be developed over the next decade [10,11]. Despite continuing improvements in the prevention of HCV transmission, and in treatment regimens, HCV is likely to persist in areas with limited access to antivirals and poor blood product hygiene and needle usage. There is evidence that significant reductions in incidence of HCV infection, particularly for non-genotype 1 strains, are unlikely without new interventions and/or a vaccine [12,13].

The characteristic of HCV that will offer the biggest problem for vaccine design is its viral variability. With sequence diversity believed to be 10 times that of human immunodeficiency virus (HIV), HCV strains are classified into 7 genotypes (numbered 1 – 7), which differ at 31 – 34% of their nucleotide positions, and which can be further divided into over 100 subtypes [14,15]. This diversity is largely due to a lack of proof-reading capacity of the viral RNA-dependent polymerase (NS5b) used by HCV during replication; therefore, HCV exists within a host as a constantly evolving population of closely related but diverse quasispecies [16].

Over the ~1000 years HCV has been infecting humans, HCV genotypes have evolved in distinct geographical regions due to "neutral" sequence drift and by rapid adaptive changes due to immunological selection pressure [17]. In recent decades epidemics of certain genotypes have spread through distinct risk groups, such as the epidemic of genotype 3a HCV amongst intravenous drug users (IVDUs) in the UK, and single-source outbreaks due to contaminated blood products [3]. An effective prophylactic vaccine for HCV will need to effectively target these prevalent circulating viral genotypes and will need to cope with HCVs' inherent mutability.

2. The immune response to HCV

Comparative analysis of individuals with distinct clinical outcomes has been performed by several groups, and there is now some consensus on the immune response required to prevent persistence of HCV, but there is no clear correlate of protection (reviewed in [18-20]). Most simply put, a strong, broad and persistent HCV-specific adaptive immune response during acute infection is required for clearance [21]; however, in the face of such adaptive immune responses HCV persists in some patients, which likely reflects the importance of other antiviral mechanisms [18,19,21].

2.1 Innate

As with other viral infections, the innate immune system – mediated by phagocytes, natural killer (NK) cells, complement and soluble antiviral factors, such as IFNs – has an important role in the control of HCV (reviewed in [22]); however, HCV has been shown to suppress early innate immune responses by multiple mechanisms, most notably by altering the downstream effects of IFN expression or by blocking its production, and by down-regulation of NK activity [23-26].

Recent genome wide association studies have highlighted the importance of innate host genes in the clearance of HCV. Single-nucleotide polymorphisms linked to the

interferon-λ3 gene have been associated with spontaneous clearance of HCV and the genotype at this locus is the most powerful baseline predictor of an SVR in genotype 1 patients treated with standard of care [27,28]; however, the overall role of IFNs in control of HCV infection remains unclear [29].

Evidently, the innate immune response is involved in the effective control of HCV, but it is more difficult to manipulate practically for use in vaccines due to its lack of specificity; nevertheless, it is clear that the use of adjuvants or vectors that elicit an innate response is key in enhancing the adaptive immune response to vaccination.

2.2 Humoral responses

Neutralising antibodies provide the clearest correlate of protection for many viral infections, and generation of such antibodies is the basis of most successful vaccines [30]. Furthermore, vaccines that generate protective antibody responses against HPV (human papillomavirus) and HBV (hepatitis B virus) demonstrate that prevention of chronic viral infection by antibody-inducing vaccination is possible [31,32]. The relevance of antibody generation for control of HCV is complex for a variety of reasons.

Circulating antibodies against structural and non-structural (NS) regions of HCV develop in all patients, regardless of outcome, and a direct correlation between viral clearance and the rapid induction of high-titre cross-neutralising antibodies has been shown, but in most patients antibody responses are not neutralising or are isolate specific [33-35]. Much of HCV sequence diversity is concentrated in areas of high variability, such as the major antibody target, hypervariable region 1 (HVR1) in E2, meaning antibodies often offer protection only against a single strain and are easily evaded by viral mutations [14,36]. If vaccine-induced immunity to HCV is to be antibody driven, a strong and broadly cross-reactive response is needed to account for extensive global diversity and inherent mutability of the virus [37-39].

Due to its hepatotropic nature, a mechanism for antibody evasion available to HCV is cell-to-cell spread via tight junctions, which are common between hepatocytes [40]. It is also likely the glycosylated coating of HCV and its interactions with high-density lipoproteins are not only used in cell entry but also hinder antibody binding [34].

A coordinated adaptive immune response involving both antibodies and T cells is normally required for pathogen control [30]. Conceptually then, is it plausible that a vaccine inducing T cells alone can prevent persistent HCV infection? Several observations suggest that it can: chimpanzees and humans can clear HCV infection without a detectable antibody response and HCV-specific cellular immune responses can be detected in exposed uninfected persons without seroconversion [34,41,42]. It has also been shown that hypogammaglobulinemic patients, deficient in antibody responses, can clear HCV [43].

HCV uses multiple mechanisms to avoid antibodies, which can explain why in the face of a detectable antibody responses HCV can persist; this combined with mounting evidence that clearance can occur without the detection of antibodies suggests that although generation of effective antibody responses would be ideal, a vaccine against HCV need not necessarily induce HCV-specific antibodies.

2.3 Cellular responses

Comparative studies have shown that a functional early T-cell response of high magnitude, targeted at multiple major histocompatibility complex class I and II epitopes, is characteristic of effective immunity, and that conversely, the hallmark of persistent infection is a weak, narrowly targeted and dysfunctional T-cell response [44-48]. Patients who go on to be chronically infected often do not lack a cell-mediated response initially, but there is evidence that the timing, persistence and functionality of the response are insufficient to control HCV [21,48,49].

Some of the most convincing evidence for the importance of T cells in protection against HCV infection comes from chimpanzee studies in which antibody depletion of CD8 T cells lead to prolonged viraemia in convalescent chimpanzees that had previously cleared two rounds of infection [50]; subsequent viral clearance was precisely correlated with a recovery of HCV-specific CD8 T cells [50]. A complementary experiment depleting CD4 T cells again led to the abrogation of a previously protective immune response [51]. Retention of an effective population of CD4 T helper cells appears to be a prerequisite for ongoing viral control, as shown by the reoccurrence of viraemia in individuals where CD4 responses wane, even after several months of apparent control [19,21,52]. Kaplan *et al.* showed in their cohort of acutely infected patients that those who cleared HCV had a highly functional virus-specific CD4 response and broadly targeted IFNγ T-cell response, but that patients with CD8 T cells or neutralising antibodies alone did not clear HCV [53]. In man, a drop in viral load and a rise in serum transaminase levels are often temporally linked to the emergence of CD4 and CD8 T-cell responses and increased intrahepatic IFNγ expression [19,48,49,51].

Single-source outbreaks have shown a clear relationship between the patients' HLA type and the outcome of their infection, with HLA-B27, HLA-B57 and HLA-A3 being associated with protective responses, again emphasising the importance of effective antigen presentation and concomitant T-cell response in the clearance of HCV [54-57].

It should be noted, however, that responses of similar breadth and magnitude to those affording protection from persistence in some patients have been seen in patients who go on to be chronically infected and there have been contrasting results when trying to correlate the magnitude of the intrahepatic cytotoxic T lymphocytes (CTL) and viraemia or outcome of infection [33,45,58].

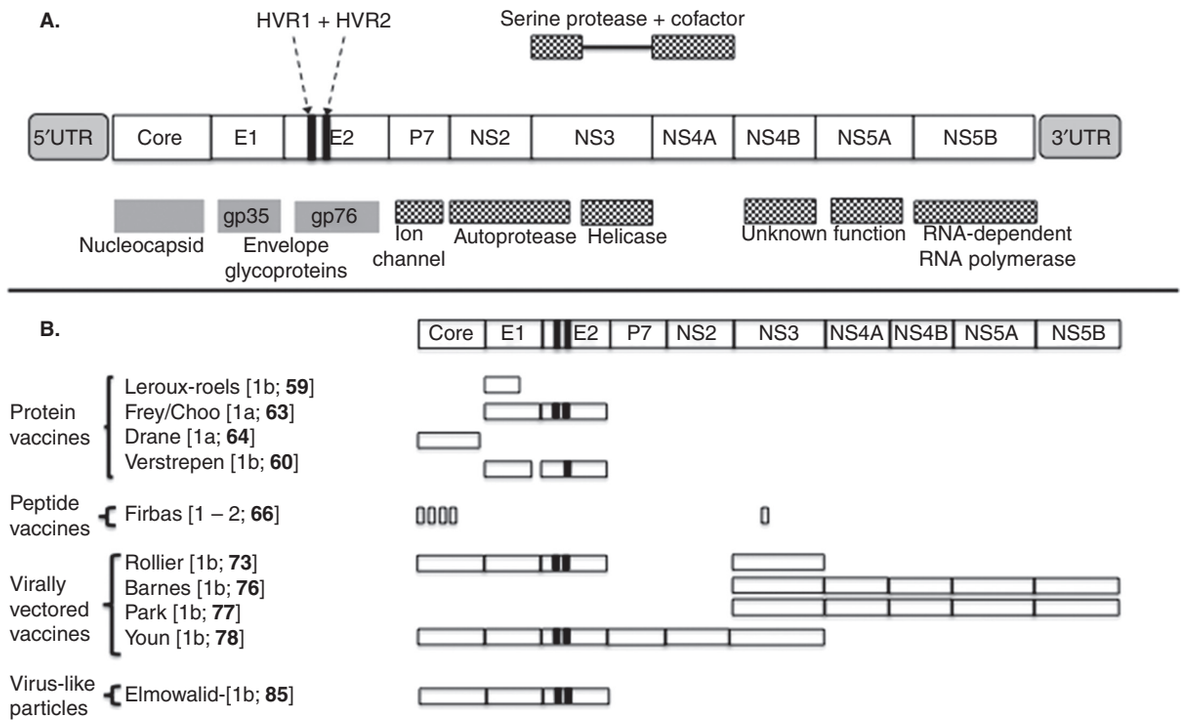

Figure 1. HCV genome structure and vaccine immunogens. (**A**) Organisation of the HCV genome: HCV, a single-stranded RNA virus of ~ 9.5 kb, consists of a single open-reading frame and two untranslated regions. HCV is transcribed as a single polyprotein, which is cleaved by a host signal protease in the structural region and the HCV-encoded serine protease in the NS region. The hypervariable regions of E2 (HVR1 + HVR2) are indicated by dashed arrows. The protein products of cleavage are shown. The structural regions consist of core and the two envelope proteins, gp35 and gp76. The NS proteins are shown and their functions are described where known. (**B**) Prophylactic vaccines for HCV tested in primates (including man) are listed according to the lead author of the paper in which they are described. The relative coverage of the HCV genome by vaccine immunogen is shown. The genotype of the immunogen encoded in each vaccine is shown in parenthesis with the paper reference [].

3. Current vaccine approaches

3.1 Recombinant protein vaccines

Several mechanisms have been investigated for the introduction of HCV-specific antigens to induce protective immunological memory (**Figure 1**; **Table 1**).

The genes encoding HCV viral proteins have been isolated and cloned into bacteria, yeast or mammalian cells, and the recombinant protein expressed purified for use in HCV vaccines. The advantage of recombinant protein vaccines is that they do not contain the pathogen or its genetic material and they do not require cultivation of the organism.

The first prophylactic vaccine candidate tested for HCV, T2S-918/InnoVac-C by Innogenetics, consisted of a C-terminally truncated recombinant E1 protein (**Figure 1**) with aluminium hydroxide (alum) adjuvant [59]. This vaccine elicited antibody titres against E1 in healthy volunteers that were significantly higher than those seen in patients with persistent HCV infection, but Innogenetics ceased work on this vaccine in 2007 [59].

The immunogenicity of E1 and E2 (with deletion of HVR1; **Figure 1**) was assessed as separate proteins, potentially uncovering new antibody targets not available in the natural heterodimeric form of E1E2 [60]. After vaccination with either E1 or E2 adjuvanted with alum, antibodies were elicited in four chimpanzees, but only antibodies against E1 were shown to neutralise HCV pseudoparticles (HCVpp) and antibody titres declined after challenge [60]. Chimpanzees were challenged with a 1b strain of HCV and only chimpanzees vaccinated with E1 were protected from viral persistence [60].

Full-length heterodimeric E1E2 has also been tested (**Figure 1**) in vaccines and proved to be highly immunogenic, showing sterilising immunity in one study against a homologous HCV strain in five of seven chimpanzees [61]. By combining results from chimpanzee vaccine studies using E1E2 glycoprotein it was shown to offer protection from persistent HCV infection in 10/12 and 8/9 chimpanzees when challenged with homologous or heterologous HCV strains, respectively [62]. When this vaccine moved into Phase I human trials, strong antibody responses were detected

Table 1. Primate and human studies describing candidate prophylactic HCV vaccines.

Type of vaccine	Investigator	Lead author	Year	Vaccine	Tested in	Adjuvant	Refs.
Recombinant protein	Innogenetics	Leroux-Roels	2004	Recombinant E1 (T2S-918/InnoVac-C)	Human n = 20	Alum	[59]
	Chiron/Novartis	Choo	1994	rE1E2	Chimpanzee n = 7	MF59	[61]
		Frey	2010	rE1E2	Human n = 60	MF59	[63]
	CSL Ltd.	Drane	2009	Recombinant Core	Human n = 30	ISCOMATRIX	[64]
	BPRC, Holland	Verstrepen	2011	Recombinant E1 or E2	Chimpanzee n = 4	Alum	[60]
Peptide	Intercell AG	Firbas	2006	7 HLA-A2 restricted peptides (IC41)	Human n = 128 (HLA-A2)	Poly-L-arginine	[66]
		Firbas	2010	7 HLA-A2 restricted peptides (IC41)	Human n = 54 (HLA-A2)	Poly-L-arginine	[67]
Virally vectored	Transgene Co.	Rollier	2007	DNA/MVA	Chimpanzee n = 4	-	[73]
	Okairos Co.	Folgori	2006	Ad6/Ad24 + electroporated DNA	Chimpanzee n = 5	-	[74]
		Fattori	2006	Ad6/Ad6/ChAd32	Rhesus macaque n = 3	-	[75]
	University of Oxford/Okairos	Barnes	2012	Ad6/ChAd3	Human n = 30	-	[76]
	NIH/Okairos Co.	Park	2012	Ad/DNA	Chimpanzee n = 5	-	[77]
	NYC blood center	Youn	2008	Recombinant vaccinia	Chimpanzee n = 4	-	[78]
Other	NIH	Elmowalid	2007	VLPs	Chimpanzee n = 4	AS01B	[85]

Ad: Adenovirus, numbers indicate type; ChAd: Adenoviruses of Chimpanzee origin; BPRC: Biomedical Primate Research Centre, Holland; DNA: Plasmids containing HCV genetic material; HLA-A2: Human leukocyte antigen serotype A2; n: Number of subjects used in study; rE1 or E2: Chimpanzees were vaccinated either with recombinant E1 protein or with recombinant E2 protein; rE1E2: Recombinant E1E2 heterodimer.

by enzyme-linked immunosorbent assay [63]; these antibodies could block viral E2 protein-binding CD81, a major entry receptor for HCV [63]. Despite these promising results technical difficulties in the manufacture of E1E2 protein have hampered its use in vaccines.

Novartis is also pursuing a vaccine consisting of recombinant HCV core protein (**Figure 1**) produced in yeast, administered with a potent T-cell adjuvant immunostimulating complex matrix (IMX) [64]. Promising results in rhesus macaques led to a Phase I dose escalation trial in 30 healthy volunteers, where all but one showed vaccine-induced antibodies against HCV core protein, but T cells were detectable in only 2 volunteers receiving a high dose of vaccine [64]. Limited by the amount of vaccine available, doses higher than 50 µg of recombinant core protein have yet to be tested.

The use of whole heat-killed recombinant yeast that express targeted molecular immunogens (tarmogens) has also been assessed (**Figure 1**) [65]. A core-NS3-5 fusion protein is encoded in the vaccine candidate GI-5005a and when combined with IMX and administered to five naïve chimpanzees a T-cell response was measurable in the liver and blood [65]. Despite altered viral kinetics during the acute phase of infection in all vaccinated animals, relative to controls, none cleared HCV after challenge [65].

3.2 Peptide vaccines

Synthetic HCV peptides have been used to induce T-cell immunity through direct presentation on antigen-presenting cells (**Table 1**). Peptide vaccines are HLA-specific and target only a selected subset of epitope sequences within HCV, limiting their breadth and coverage within the population but allowing closer control over the immunodominance hierarchy of vaccine responses.

Five synthetic HCV peptides containing T-cell epitopes (**Figure 1**), administered with poly-L-arginine, make up the vaccine candidate IC41. When administered to 128 HLA-A2+ healthy volunteers in a Phase I study, IC41 was shown to be safe and immunogenic [66]. Few IFNγ-producing cells were induced by IC41, as measured by IFNγ ELISpot (**Figure 2**; median of 30 spot-forming cells [SFCs] per 10^6 peripheral blood mononuclear cells [PBMCs]) [66].

A further study testing the efficacy of this vaccine when administered subcutaneously or intradermally showed 65 – 100% of vaccinated healthy volunteers had lymphoproliferative responses to HCV proteins but again weak T-cell responses (**Figure 2**) [67]. The use of the TLR7 agonist, Imiquimod, had no effect on vaccination [67]. In the setting of chronic infection this vaccine caused a significant 0.47 log 10 drop in HCV RNA, but this did not correlate with the size of the T-cell response [68,69]. The biotechnology company Intercell AG aims to enhance this approach by broadening the number of epitopes targeted and by investigating new adjuvants.

3.3 DNA vaccines

Injection of recombinant plasmids has been shown to result in effective protein expression *in vivo* and a subsequent immune

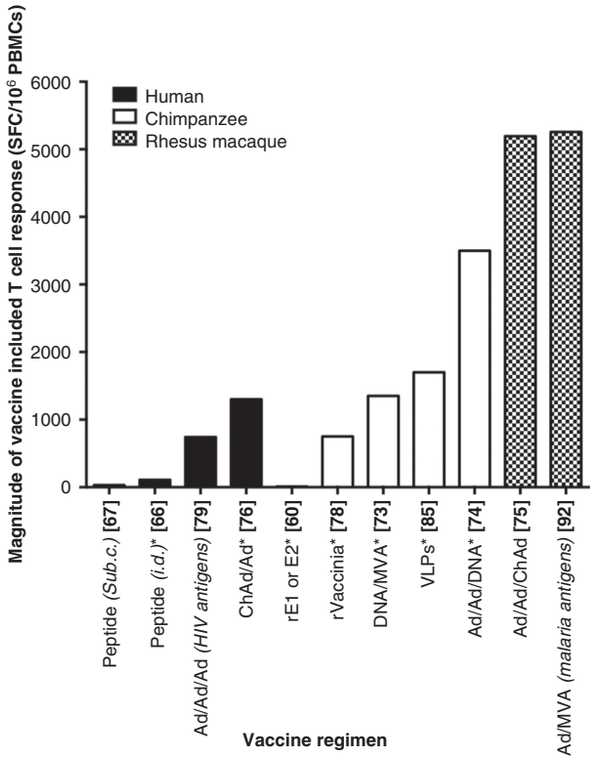

Figure 2. Magnitude of T-cell response to vaccination. A comparison of the magnitude of vaccine-induced T-cell responses in primates is shown (median of peak response after vaccination as measured by IFNγ ELISpot). Vaccines contained HCV antigens unless otherwise stated. Successive vaccinations are separated by a forward slash, e.g., DNA/MVA refers to a vaccine regimen using DNA priming followed by an MVA boost. References in parenthesis [].

* Denotes approximation of published values. ChAd: Adenovirus of chimpanzee origin; i.d.: Vaccine was administered intradermally; rE1 or E2: Chimpanzees were vaccinated either with recombinant E1 protein or with Recombinant E2 protein; rE1E2: recombinant E1E2 heterodimer; Sub.c.: Vaccine administered subcutaneously; rVaccinia: Replication competent recombinant vaccinia vector.

response in mice, but this initial success has not yet been translated well into man. DNA uptake and gene expression decrease with the size of the immunised host; however, there has been much development in technologies to improve cell transfection, such as transdermal delivery with the gene gun, and *in vivo* electroporation (reviewed in [70]).

Plasmids encoding HCV NS3/4a (ChronVac-c) or core/E1/E2 (CICGB-230) have shown some efficacy as potential therapeutic vaccines for HCV, but there is no published data on their effectiveness as prophylactic vaccines [71,72].

3.4 Vector-based vaccines

Over the last decade great advances in molecular virology have enabled the manipulation of viruses for delivery of foreign genetic material to mammalian cells (**Figure 1**; **Table 1**) [73-79]. Their highly evolved mechanisms for cell entry and gene expression within the host cell remain intact and viral vectors can be rendered non-pathogenic and non-replicative by deletions at specific locus [80]. Some viral vectors at the earliest stages of testing as delivery vehicles for HCV genetic material include alphaviruses, canary pox, ovine atadenovirus and semliki-like viral particles [81-84].

Virus-like particles (VLPs) are attractive vectors for gene delivery as they mimic the properties of native virions, are safe and are easily manufactured. VLPs encoding the HCV core-E1E2 genes (**Figure 1**) induced a large HCV-specific T-cell population in chimpanzees (**Figure 2**) and all four vaccines cleared challenge with homologous strain of HCV [85]. Surprisingly, no HCV-specific antibody response was detected [85].

Adenoviral (Ad) vectors are the best characterised viral vectors and have emerged as the most potent at T-cell priming in non-human primates (NHPs) and humans [86]. Ad-based vaccines are particularly attractive gene vehicles as they can stably express large foreign inserts (~10 kbp), they remain epichromosomal and can be easily rendered replication defective by deletion of the E1 locus [80,87].

The major limitation with the use of adenoviruses is that pre-existing immunity to the vector can lead to its clearance before a response is elicited to the inserted immunogen [86]; this can in part be overcome by the use of rare subtypes that are of low seroprevalence, or the use of adenovirus with altered surface proteins [86]. An extensive study of chimpanzee adenoviruses by researchers at Okairos (Rome, Italy) isolated over a thousand strains and showed that their immunogenicity in mice varied widely [88]. Chimpanzee adenovirus 3 (ChAd3) and Ad6 were selected for analysis in human and animal trials, with the whole NS region of HCV (genotype 1b, BK strain) [76].

Support for the use of Ad-based vaccines to induce protective T-cell populations in man came from a therapeutic trial in which chimpanzees received heterologous Ad-Ad followed by further boosting with electroporated DNA, all containing the NS region of HCV (**Figure 1**) [74]. All vaccinated chimpanzees produced a high-magnitude T-cell response (**Figure 2**; peak total anti-HCV responses ranged from 615-2509 SFC/10^6 PBMCs and 1108-7678 SFC/10^6 PBMCs for CD4 and CD8 T cells, respectively) and when challenged with a heterologous HCV strain four of five cleared the virus [74]. Vaccinated animals showed strong anamnestic responses that resulted in a blunted peak viraemia and shorter period of infection relative to controls [74]. Prime-boost vaccination with heterologous adenovirus containing HCV NS also induced a large and broadly targeted HCV-specific T-cell response in rhesus macaques (**Figure 2**) [75].

On the strength of this preclinical data the Ad vectors ChAd3 and Ad6 were tested in a Phase I clinical trial in healthy volunteers [76]. All 10 patients receiving the highest dose of Ad responded to vaccination, with peak T-cell responses averaging over 1000 SFC/10^6 PBMCs (**Figure 2**; range 443 – 4263).

However, boosting with heterologous Ad in healthy volunteers was not as effective as predicted from the results in rhesus macaques, which is likely due to higher levels of cross-reactive antibodies against the Ads in humans [75,76].

Modified vaccinia Ankara (MVA) is another attractive vaccine vector due to its excellent safety record and immunogenicity in man. It has been shown to be particularly effective as a boosting vector, broadening and increasing the magnitude of pre-existing T-cell responses [86,89-92].

A heterologous prime-boost regimen with DNA and MVA encoding core-E1-E2 (**Figure 1**) and NS3 was tested in naïve chimpanzees by TRANSGENE [73]. A large T-cell response was seen post-vaccination by IFNγ and IL-4 ELISpot (**Figure 2**), but proliferative responses were transient and a high expression of IDO, CTLA-4 and PD-1 on HCV-specific T cells after challenge suggested that induced T cells could have been dysfunctional [73]. Three of four went on to be chronically infected when challenged with a heterologous J4 strain of HCV [73].

Using the malaria antigen ME.TRAP, priming vaccination with Ad elicited similar T-cell responses to Ad encoding HCV NS, and these responses were boosted 3.1 – 5.2-fold by vaccination with MVA containing ME.TRAP (**Figure 2**) [92]. Building on the results outlined above, a trial to assess the safety and efficacy of a prime-boost regimen using ChAd3 boosted with MVA encoding HCV NS in patients and healthy volunteers is ongoing [93]. This Ad/MVA vaccine regime has also progressed to a Phase II study, which will take place in an intravenous drug using community in Baltimore [94]. This study will be the first double-blinded, randomised, placebo-controlled trial of a vaccine to prevent HCV persistence. The trial will enrol 350 subjects and is set up to assess whether this regimen can enhance the rate of spontaneous resolution of HCV infection.

A single meta-analysis has compared the overall outcome of HCV infection between differing prophylactic vaccine studies in chimpanzees, and also performed a comparison between naïve, re-challenged and vaccinated animals [5]. The authors show that immunological memory after prior viral clearance, or vaccination, leads to a reduction in peak HCV RNA titre and increased HCV clearance rates after viral challenge [5]. Additionally, the study dissects the outcome of infection after vaccination according to whether or not the vaccine included NS or structural regions of HCV, concluding that vaccines containing only structural antigens were more efficacious [5]; however, these data should be interpreted with caution, since a large proportion of animals were vaccinated with the E1E2 recombinant protein heterodimer (Novartis; [62,95]), and differences in the challenge strain and dose of virus between studies complicate the comparative analysis [5]. Examples of protective and non-protective vaccines can be found for vaccines containing structural antigens, NS antigens or both, and more research is needed into which antigens offer the best protection [73,74,78,95].

4. What is a protective T-cell response?

Several methodologies are progressing to clinical studies in humans but what do we know about the type of immune response offering protection from persistent HCV infection, and how do responses to the current vaccine candidates measure up against this ideal?

4.1 Magnitude

The most fundamental characteristic of a T-cell response is its magnitude, as measured directly by the frequency of antigen-specific T cells or by the number displaying a certain effector function, most commonly production of IFNγ.

Although no defined cut-off for a protective response against HCV exists, responses in individuals who clear acute infection are typically in the region of a few hundred IFNγ-producing cells per million PBMCs, and the responses often remain detectable for many years after infection [21,53,74,96,97].

Prospective vaccines against HCV have induced T-cell populations with a wide range of magnitudes (**Figure 2**). One of the few vaccines tested in humans for which T-cell responses were measured, IC41, had a median response of only 30 SFC/10^6 PBMCs (range 15 – 185) [67]. The largest magnitude HCV-specific T-cell responses seen in animal models and humans have come from regimens using Ad vectors (**Figure 2**). The responses to Ad6-ChAd32 heterologous prime-boost had an average of 4924 SFC/10^6 PBMCs in rhesus macaques and 1202/1400 SFC/10^6 PBMCs in the two Ad-Ad regimes tested in humans (**Figure 2**) [75,76].

Although vaccines that induce high levels of HCV-specific T cells in animal models have protected against persistent HCV infection, this is not always the case; T-cell responses as high as 2368 SFC/10^6 PBMCs in chimpanzees vaccinated with DNA and boosted with MVA were not protective against challenge with a heterologous strain of HCV, highlighting the importance of other factors, in particular the functionality and antigen specificity of the vaccine-induced T cells [5,73].

With the development of viral vectors we now have the means to induce large numbers of antigen-specific and IFNγ-producing T cells. However, it is apparent that the magnitude of the T-cell response alone is a poor predictor of protection for many viral infections and that an understanding of the combination of functions possessed by T-cell populations is needed [91,98-101]. Several T-cell parameters of importance for vaccine design are discussed below.

4.2 Breadth

The selection pressure exerted by the adaptive immune response rapidly selects for escape variants, leading to persistence of HCV strains that are unrecognisable by circulating T cells and antibodies in both natural infection of humans and in animal infection models [36,102-104]. The breadth of a T-cell response has been reproducibly associated with control in human correlative studies [21,45,96,97]. For an effective HCV vaccine, a T-cell response targeting multiple epitopes may

be required to limit the possibility of viral escape and to effectively block viral replication [45,96,97].

The breadth of the T-cell response to vaccination is dependent upon the size and specificity of the immunogen (**Figure 1**), and vaccine studies to date have induced T-cell responses with a wide variation in breadth. The breadth of the response using a peptide vaccine will be restricted to the HLA repertoire of the vaccinated population, whereas a virally vectored vaccine approach may induce a broadly targeted T-cell response on a diverse HLA background population [66,76].

As well as targeting multiple HCV epitopes, an effective global vaccine will also need to target multiple viral genotypes [14]. Using HCVpp recombinant E1E2 protein vaccines induced antibodies with cross-reactivity against genotypes 1a, 1b and 2a [105]. Barnes *et al.* showed that response to Ad-Ad vaccination containing the NS region from a 1b BK strain recognised peptides from genotypes 1a, and to a more limited extent, 3a, the most prevalent genotypes in Europe and America [76].

4.3 Proliferation and cytokine production

A dysfunction in the T-cell proliferative capacity has been repeatedly identified in those who fail to control HCV relative to those who clear [46-48,106]. Good proliferative capacity is key for memory responses induced by prophylactic vaccination, as a rapid and large expansion of secondary effector T cells is needed during recall responses. Lymphoproliferative responses to HCV proteins have been seen in several HCV vaccine studies [59,63,66,74,76,85].

Intracellular cytokine staining is used to assess the production of several cytokines relevant to viral control by antigen-stimulated T cells. The production of IFNγ and TNFα is often measured due to their direct antiviral effect, as well as IL-2, which promotes the clonal expansion of T-cell populations on activation and influences differentiation of T-cell subsets. These cytokines are often measured in conjugation with CD107α (lysosome-associated membrane protein-1), a marker of T-cell degranulation, and the pro-inflammatory cytokine MIP-1-β (macrophage inflammatory protein-1β; CCL4).

It has been assumed, but not convincingly shown, that a polyfunctional T cell, with the capacity to carry out multiple antiviral functions, is more effective at clearing a virus than a monofunctional T cell; evidence of this came from comparative studies of long-term non-progressors (LTNPs) and progressors in the setting of HIV infection, and mouse studies of *Leishmania major* [99,107]. The T-cell population in LTNPs, relative to progressors, is enriched for single CD8 T cell that can co-produce MIP-1-β, TNFα, IFNγ and CD107α [98]. But these highly polyfunctional T cells are lacking in some LTNPs, when present represents a small fraction of the total HIV-specific T-cell population, and could simply be an indicator of low antigenic load in these individuals [98,107]. Polyfunctionality not only affords a larger repertoire of functions for the individual cell, but it can also mean a larger per-cell production of the cytokine relative to monocytokine producers (e.g., in some studies monocytokine-producing T cells made 10× less IFNγ per cell than polyfunctional T cells) [99,108-110].

Evidence of a hierarchy in cytokine production is emerging, where increased antigen exposure and co-stimulation lead to an increase in functions expressed by a T cell [111-114]. Viola *et al.* showed that MIP-1-β and IFNγ are most readily released by T cells on limited stimulation and that IL-2 production is only triggered when a T cell has been exposed to high levels of antigen and co-stimulation [114]. The antigenic load resulting from vaccination is highlighted as a key attribute that will affect T-cell quality and vaccine efficacy. This hierarchy is evident in vaccine responses elicited by Ad-Ad and Ad-MVA vaccination, and polyfunctionality is a characteristic of T-cell responses to these vector combinations [76,92].

4.4 Cytotoxicity

One functional attribute of human CD8 T cells that unequivocally combats acute viral infections is cytotoxicity [21,115]. Direct cytotoxic killing of infected cells is primarily mediated by the activation of apoptotic pathways within the target cell by granzyme cleavage of intracellular caspases [116]. On recognition of an infected cell, activated CTL secrete lysosomes containing the pore-forming protein perforin and granzymes, which are delivered to the target cell inducing its apoptosis [117,118].

Rather than its *ex vivo* expression, the rapid up-regulation of perforin is a key effector function of T cells, and one that appears to be lacking in HCV-specific T cells taken from chronically infected patients [98,119,120]. Although it has been shown that during chronic infection HCV-specific CD8 T cells are often deficient in perforin expression and up-regulation relative to CMV-specific populations, it remains unclear how much perforin is necessary to initiate granzyme-mediated killing [120].

Surface mobilisation of CD107α has been used to identify cells that have degranulated on peptide stimulation and, although this assay does not show killing directly, or give information on the content of the granules released, it can be combined with staining for granzymes and perforin to give an indication of the cytolytic potential of a T cell [121].

Few trials have assessed the cytolytic capacity of vaccine-induced CTL, but it remains a key parameter that should be assessed in any vaccine aiming to mediate protection through induction of T cells. After vaccination with heterologous Ad-Ad encoding the NS region of HCV, the vast majority of HCV-specific T cells express both granzyme A and B, and often high levels of perforin [76]. It was also shown that these cells produced CD107α on peptide stimulation, showing that they have the capacity to kill HCV-infected cells [76].

4.5 Phenotype

Research over the last decade has revealed an ever increasing complexity and division of labour within T lymphocytes, particularly within the memory compartment [101,122-124].

Antigen-experienced cells in humans express the short, CD45RO, form of the protein tyrosine phosphatase CD45, and the long form, CD45RA, is expressed by naïve T cells and is re-expressed by a subset of CD8 T cells [125]. In combination with the lymph node homing receptor CCR7, four broad populations of T cells can be described [122]: Central memory T cells (Tcm; CD45RA-, CCR7+) that home to the lymph nodes and have limited effector function but high proliferative capacity; Effector memory T cells (Tem; CD45RA-,CCR7-) that show immediate effector and cytolytic function and circulate peripheral tissues; naïve, antigen-inexperienced T cells (CD45RA+, CCR7+); "terminally differentiated" effector memory T cells (Temra; CD45RA+, CCR7-) that have re-expressed CD45RA.

Additional complexity has been found in mice studies that have tried to identify precursors of long-lived memory cells in the effector pool. Effector T cells have been divided into memory precursor effector cells and short-lived effector cells by their expression of CD127 (IL-7Rα), and KLRG1 and a population of T cells with stem cell-like properties have been described (Tscm) [126-128].

Rather than being a trivial pursuit, the description of these subsets of T cells has led to the identification of certain subsets as being the main mediators of protection after vaccination [91,129]. An Ad-MVA heterologous prime-boost regimen encoding malaria antigens preferentially induced antigen-specific Tem that mediated protection against challenge with malaria sporozoites, as confirmed by transfer experiments [91]. Tem were also shown to mediate protection against SIV challenge in rhesus macaques [129].

It is now clear T cells play a key role in clearance of HCV, but we are lacking information about which subsets mediate this control and which subsets we should aim to elicit by vaccination. It is likely that a mixed population of lymph-node homing Tcm, with the potential to rapidly proliferate and differentiate into effector T cells, along side a population of Tem, which circulate the periphery and have a more immediate effector function, would in theory be most effective.

The vaccine platform used to deliver immunogens has a profound influence on the type of T-cell response elicited, due to differences in the innate signalling pathways stimulated and the persistence and amount of antigen after vaccination [86,91,129,130].

Low levels of transcriptionally active Ad have been shown to persist long term at the site of vaccination, in the liver and in lymphatics, after vaccination with Ad in mice and primates; however, transgene expression by MVA becomes undetectable after ~ 2 days [131]; this may explain why MVA vaccination is characterised by the induction of Tcm, whereas a single Ad induces and maintains an active Tem population, as well as developing Tcm and Temra [91,131]. It is clear the number of antigen encounters, or vaccinations, also influences the extent of T-cell differentiation [132]. For example, repeated stimulation by antigen can lead to a larger population of Tem and Temra but fewer Tcm [133].

Barnes *et al.* describe the phenotypic properties of T cells induced by heterologous Ad-Ad vaccination encoding the NS region of HCV [76]. Vaccine-induced CD8 T cells peaked in magnitude 2 weeks post Ad priming, and subsequently contracted to a memory T-cell population in which a significant proportion had down-regulated PD-1, and re-expressed CD127 [76]. A key feature of the CD8 T cells induced by Ad encoding the NS region of HCV is the re-expression of CD45RA, giving a mixed population consisting of Temra > Tem > Tcm [76]. This phenotype is remarkably similar to that of the T cells induced by the highly efficacious vaccines for yellow fever and smallpox (Dryvax), which have excellent safety records in humans and elicit large T-cell responses that are associated with life-long protection [134].

The use of classic phenotypic markers has under-represented the complexity of T-cell phenotypes, but the advent of sophisticated cytometric techniques (i.e., multiparametric flow cytometry, cytometry by time-of-flight, and gene expression profiling) capable of analysing multiple T-cell parameters simultaneously may enhance the stratification of T-cell subpopulations by function [101,130,135]; for example, a recent study described phenotypically identical T-cell populations induced by different vaccine regimens that were distinct when the cell transcriptome was assessed [130].

Three prime-boost regimens encoding HIV Env gene were compared by Flatz *et al.* (DNA-Ad, Ad-Ad and Ad-rLCMV [recombinant Lymphocytic choriomeningitis virus]) and despite inducing T-cell populations that were similar in magnitude, cytokine production and Tem/Tcm phenotype, the gene expression profile of the cells induced by different regimens was distinct [130]. Using linear discriminant analysis, antigen-specific T cells clustered separately for each regimen and there were noticeable differences in expression in senescence and homing markers (e.g., Eomes, CCR7, CXCR3, CCR5, KLRG1 and Klrk1) [130]. Therefore, even using the magnitude, cytokine production and classic phenotyping may not be sufficient to identify correlates of protection because they are insensitive to the full extent of heterogeneity in CD8 responses [130].

Clearly, a better understanding of the division of labour between T-cell subsets and the steps involved in T-cell differentiation and memory formation should help identification of correlates of protection for viral pathogens.

5. Conclusion

The development of a vaccine capable of preventing chronic HCV infection remains the most cost-effective and realistic method of controlling HCV globally, and it would find a target population in at-risk groups in developed countries and in entire populations in many developing countries [13,136].

The prospects for a HCV vaccine have improved greatly in the last decade and there is now strong evidence that HCV is highly amenable to a prophylactic T-cell vaccine. Evidence from secondary infections in patients and from vaccine studies

in humans and NHP show that immunological memory can protect against persistence of HCV and therefore disease, and this protection appears to be prominently mediated by cellular immunity [4-6,137].

We now understand that clearance of HCV can occur without the induction of a measurable antibody response; however, vaccine-induced neutralising antibodies, when targeting circulating virus, also show effective control of HCV [60,95]. It is likely that a T-cell-based vaccine will show enhanced efficacy when combined with a vaccine that induces cross-reactive antibodies.

With the development of novel DNA delivery systems, viral vectors and VLPs we now have the tools to induce large antigen-specific T-cell populations (**Figure 2**) and we are developing adjuvants to enhance these further and to tailor the types of T-cell subsets induced by vaccination.

We now know that different subtypes of T cell exist, with different functionalities, and that these subsets offer different levels of protection against specific pathogens. As this understanding develops further we will be able to better asses and manipulate vaccine-induced T-cell populations. To do this it is essential that we better describe the T-cell parameters associated with control of HCV.

6. Expert opinion

A vaccine for the prevention of HCV infection would address a huge unmet clinical need globally. In developed countries we would envisage that an effective prophylactic vaccine would be administered to at-risk populations, but in large areas of Africa and Asia, where prevalence rates are high, a universal vaccination strategy would be optimal. It is this need that has driven an intense research program in HCV vaccinology over the last 15 years.

Detailed studies of natural infection have shown that complete HCV viral control is attainable through effective host anti-viral immunity. Multiple lines of evidence point to the crucial role played by host T-cell immunity in viral control, in addition to host innate immune genes. Effective humoral immunity has been less clearly associated with protection – nevertheless, a vaccine that induced appropriate B-cell responses may improve vaccine efficacy. Over the last decade numerous vaccine strategies have been assessed in small animal and primate models. Broadly these include HCV envelope protein vaccines designed to generate protective humoral responses, peptide vaccines that are restricted by host HLA, and more recently DNA and virally vectored approaches (**Figure 1**). DNA vaccination is capable of generating robust T-cell immunity, but requires additional delivery mechanisms such as electroporation. Virally vectored vaccines have recently reached Phase II studies and show immense promise when used alone, or in the future alongside other approaches (**Figure 2**).

To date, the exact correlates of immune protection against persistent HCV infection have not been clearly defined, and indeed this may never be achieved given the heterogeneity contained within small study populations of patients with primary infection and the real limitations of animal models of HCV infection. However, in broad terms, the generation of $CD4^+$ and $CD8^+$ T cells that target multiple HCV antigens at a high magnitude, and that persist over months to years, is seen in humans that control viraemia after primary infection, and also in primate prophylactic T-cell vaccine studies [44-48,74,75,77,78]. Therefore, current vaccine strategies should at least aim to recapitulate these findings in Phase I human studies before efficacy testing. Ideally a potent T-cell vaccine would be combined with an antigen capable of generating broadly cross-reactive neutralising antibodies against HCV envelope. However, currently this is not technically feasible.

Current vaccine approaches are capable of inducing T-cell immunity of a breadth and magnitude that has been associated with viral clearance in humans. However, we do not know if these T cells possess all the "qualities" that are required for long-term viral control, since these qualities are not absolutely defined. This presents a real limitation in the design, assessment and ranking of vaccine candidates. Natural history studies that identify T-cell parameters associated with viral control in the context of HCV and also other pathogens will contribute to our understanding in this area (**Figure 3**). However, Phase II studies of efficacy testing in humans may be required to further define immune correlates of protection, for optimal vaccine generation in the laboratory – a process termed "reverse vaccinology" (**Figure 3**).

We believe that in order to now advance the field, comparative Phase I studies in human trials that address the magnitude, breadth and functionality of T and B cells induced by leading vaccine candidates, incorporating a range of immunogens, should be developed. Ultimately, Phase II studies assessing efficacy of the most promising regimens will be required. Since efficacy studies are logistically challenging, consortiums of investigators that care for well characterised at-risk cohorts (e.g., IVDUs and men who have sex with men) could be established now. If this approach is taken then a platform will be in place for efficacy testing of the most promising vaccine candidates when required (**Figure 3**). These studies would assess the change of incidence of primary HCV infection that progresses to chronic disease. A placebo-controlled, Phase II study assessing vaccine efficacy using these principles in IVDUs is currently underway in Baltimore, USA, and provides proof of principle that this kind of study is now feasible [94]. This approach can move forward in parallel with laboratory studies that aim to improve small animal models of HCV and to further define the correlates of immune protection in natural history studies.

Another challenge in the field is the generation of cross-reactive immune responses that are capable of protecting people from diverse HCV strains. This may be less of an issue in populations where only one viral genotype commonly

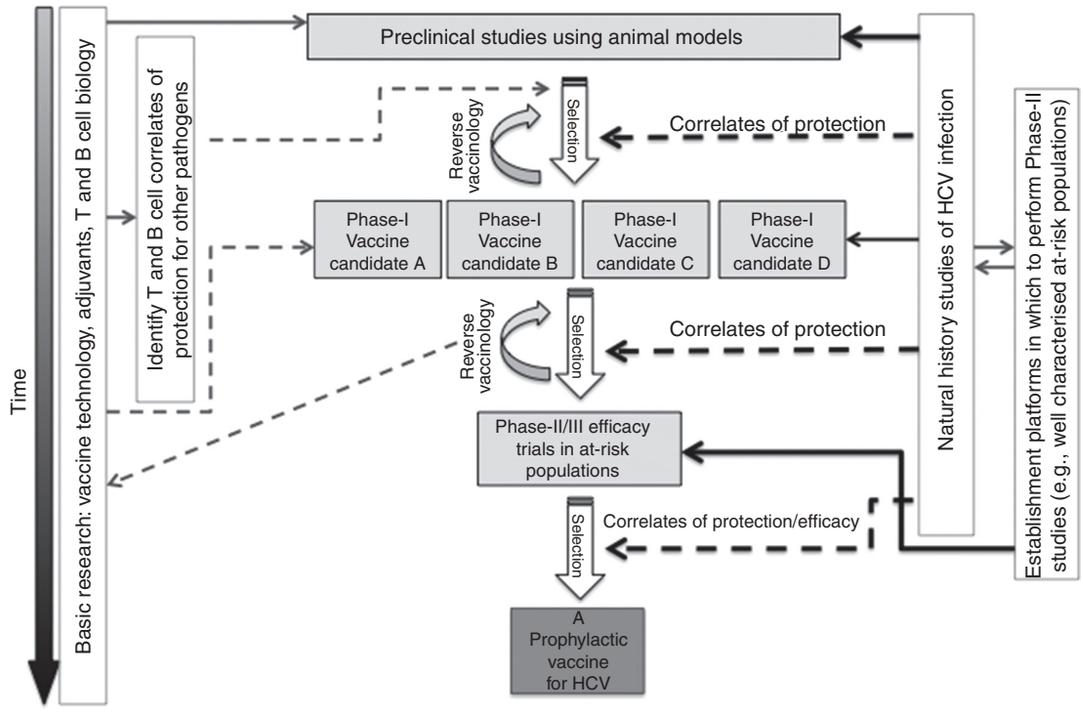

Figure 3. Progress to an effective prophylactic vaccine against HCV. A summary of some of the key interactions between basic research and vaccine studies is shown. Natural history studies of HCV infection are used to better understand the immune correlates of protection against HCV infection; this understanding informs all levels of vaccine design, in particular the design of vaccines for preclinical and Phase I studies and at the selection stages when assessing efficacy of candidate vaccines. Cohorts of at-risk populations need to be characterised before candidate vaccines can be tested in Phase II/III studies and so this work should be done in parallel with early-stage vaccine assessment. Phase II/III studies of vaccine efficacy may be required to further define correlates of protection for optimal vaccine generation – a process termed "reverse vaccinology". Basic research into vaccine modalities, adjuvants and the biology of T and B cells can be fed into the process of vaccine development at all stages to allow us to better design, assess and implement novel vaccines.
HCV: Hepatitis C virus.

circulates, though even in this case there is considerable diversity between individuals infected with the same HCV genotype. However, in many countries multiple viral genotypes circulate, for example in the United Kingdom approximately 50% of people are infected with genotypes 1a or 1b and 50% with genotype 3. Ideally then, an effective vaccine would be capable of protecting from multiple HCV strains. This may be achieved through administering more than one genotype-specific vaccine at a population level, or through the design of immunogens within a single vaccine that target multiple genotypes.

The HCV research community can be proud of the progress made over the last two decades in understanding HCV pathogenesis through *in vitro* replication models, and in the recent advances in the treatment of HCV. In the next decade we will see the implementation of multiple new DAAs that will cure many of those already infected. However, these treatments will come at a significant financial burden, and will be unavailable to most, either because infected people exist in resource poor settings, or because people are unaware that they are infected. Furthermore, these drugs are least effective in people with advanced disease who need them most. For these reasons we believe that the adage "prevention is better than cure" holds true for HCV today.

This, combined with new vaccine technologies that are really capable of delivering potent anti-HCV viral immunity, argues for major new investment in HCV vaccine Phase I, and efficacy studies. This investment will require not only significant financial resourcing, but also appropriate infrastructure and collaborative working between investigators that care for carefully characterised patient and "HCV at-risk" cohorts, across national boundaries. If these practical steps are taken, a preventative vaccine for HCV can be achieved.

Declaration of interest

E Barnes is funded by the Medical Research Council (MRC) UK, the Oxford NIHR Biomedical Research Centre and the James Martin School. Leo Swadling is funded by the MRC UK and Paul Klenerman is funded by the MRC UK, the

Oxford NIHR Biomedical Research Centre and the James Martin School. The authors are currently working on Phase I HCV vaccine studies using adenoviral and MVA vectors in collaboration with an industrial partner Okairos. The authors have no other relevant affiliations or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilised in the production of this manuscript.

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