Hepatitis C Vaccine Landscape - Executive Summary

Aim, Scope and Limitations of this Review

The research landscape dedicated to finding an HCV vaccine is vast, with many vaccine candidates in preclinical but only two strategies tested in clinical stages of development. While it is challenging to ascertain the exact number of labs working on a potential vaccine, it is safe to say that there are dozens of them around the world. It is a global research endeavor, characterized by collaborative efforts and many international consortiums and collaborations are dedicated to the advancement of HCV vaccine development. This reality often blurs the lines of singular attributions to a vaccine strategy, as most research groups concurrently investigate several adaptations of a candidate or entirely different candidates in parallel.

Consequently, the majority of review literature rightfully centers on specific mechanisms of a vaccine, and outlines their unique developments. Navigating this intricate web of research and discerning the interconnected streams that feed into each vaccine strategy can be difficult. Here, I aim to ease this challenge. By providing a general introduction to the key concepts, by detailing the core mechanisms and the current progress of today's HCV vaccine strategies, this document is compiling information about the involved contributors, stages of development, future prospects, and potential obstacles that could hinder the progress of the vaccine candidates that we know about.

As an overview, this review is limited to prominent vaccine candidates under active investigation as of 2023 and those demonstrating potential to advance towards clinical trials. Notably, it excludes research efforts focused on exploring specific characteristics of HCV antigens, provided they do not directly contribute to the development of an actual vaccine candidate. The intention is to furnish readers with a comprehensive overview of the current state of HCV vaccine candidate landscape.

Comments, additions or questions are welcome. Please email to:
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Introduction and Background

Global Impact and the Need for an HCV Vaccine

Hepatitis C virus (HCV) represents a significant global health problem, with an estimated 58 million people infected and 290,000 fatalities in 2019 alone [1]. The introduction of direct-acting antivirals (DAAs) has bolstered cure rates, yet numerous challenges such as delayed diagnosis, prohibitive treatment costs, and a decline in treatment rates stand in the way of the global eradication of HCV [1–3]. Despite a concerted global effort to outpace infection rates with cure rates, approximately 60% of countries reported more infections than cures in 2016. By 2018, only 11 out of 45 high-income countries were achieving the WHO's infection-reduction objective [2,4,5].

While DAAs are highly effective, they fall short of reversing the progression of liver disease or preventing reinfection with the virus, and a subset of patients might develop resistance [6,7].

Particularly concerning are the high reinfection rates among at-risk populations [8–14]. The high cost of DAA therapy and lack of robust surveillance programs disproportionately impact low-to-middle income countries [15,16] but even within the US, timely treatment is accessed by a mere third of insured patients with HCV [17]. The ongoing COVID-19 crisis has compounded this issue, further curtailing access to HCV diagnosis and treatment services [1,18].

The prospect of a vaccine could offer a viable, cost-effective solution, particularly for at-risk groups and those in developing nations [19,20], yet it is seen as unappealing by the pharmaceutical industry [21]. Given the grim prediction of viral hepatitis emerging as a leading global killer by 2040, prioritizing the development of an HCV vaccine is paramount [22].

General Introduction to Vaccine Immunology

In general terms, a viral vaccine contains one or more immunogens, which have sufficient structural similarity to components of the virus that the immune system recognises the virus as the same entity, and often an adjuvant, which stimulates inflammation around the site of administration so that the immunogenic components are recognised as a threat [23]. The vaccine should be highly immunogenic (i.e. stimulate a strong and persistent immune response against the virus if subsequently encountered), and ideally confer lasting immunity [24].

Infection with a live virus stimulates two kinds of adaptive immune responses: a humoral response and a cell-mediated response. The humoral response aims to recognise viral particles outside cells and prevent them from entering and infecting cells (“neutralization”), generally through the production of antibodies by cells in the B cell lineage which bind to specific sites and impair their function. The cell-mediated response aims to identify and destroy infected cells to prevent them from producing more viral particles, achieving this through a number of specialized cell types (particularly cytotoxic T cells) [24].

The means by which a vaccine’s immunogenic components are delivered affects stimulation of the humoral and cell-mediated responses, and recent years have seen a great increase in the use of nucleic acids in place of proteins. With nucleic acid vaccines, protein antigens are synthesized by host cells as they would be during a true viral infection. This necessitates delivery into cells, which can be achieved by a variety of platforms. For example, messenger RNAs (mRNAs) encoding the desired immunogens can be delivered *via* synthetic nanoparticles, or another virus can be adapted as a vector by inserting sequences coding for the immunogens into its genome [25]. Viral vectors used in human vaccines are generally designed to be replication-deficient.

In the extracellular space, immunogenicity can also be enhanced by multiple presentation of antigens on nanoparticles rather than individual antigens dissolved in solution, mimicking the concentration of antigens on the surfaces of viral particles. Conjugation of the immunogen with proteins that self-assemble into nanoparticles, such as ferritin, can be used for this purpose.

Key Components and Mechanisms of the Hepatitis C Virus Replication

The hepatitis C viral particle (virion) is composed of an outer lipid bilayer envelope with E1E2 heterodimers on its surface, which surrounds a proteinaceous structure known as the capsid, which

itself contains the virus's genetic material. Additionally, in infected individuals HCV is physically associated with lipoproteins, giving the particles (aka lipoviral particles or LVPs) a low and heterogeneous buoyant density [26,27]. The HCV genome comprises a single-stranded, positive-sense RNA (ssRNA(+)) of approximately 3000 amino acids, flanked by 5' and 3'-terminal untranslated regions (UTRs). The genome encodes functional proteins of two categories: structural proteins (core protein, which makes up the capsid, and E1 and E2, which are envelope proteins) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The role of the p7 protein is still uncertain [28], but it likely aids the viral assembly process together with NS2 [29], while NS3 [30], NS4A [30], NS4B [31], NS5A [32], and NS5B [33] form the replication complex on the host ribosome. However, most HCV proteins have multiple functions. As an example, E2 and the heterodimer it forms with E1 are not only crucial for cell surface binding, but have also been identified to facilitate escape from endosomes after internalization into the cell by binding and fusingthe viral and cellular membranes [34].

While a large number of cell surface molecules and host factors may contribute to HCV cell entry [35,36], a key player in this process is the transmembrane protein CD81 [37]. The binding site of the HCV E2 protein to CD81 is well characterized [37–39], making this receptor binding domain a key target for vaccine development.

During viral replication in the host cell, specifically in the process of translation, the virus is cleaved by host cell [40] as well as viral [41] proteases. The envelope proteins E1 and E2 then enter the lumen of the ER, embed themselves into the membrane, and become glycosylated [42], a biological process where carbohydrates are attached to proteins.

Challenges in Developing an HCV Vaccine

HCV's genome has exceptional genetic variation due to its error-prone RNA polymerase [43], with circulating strains currently including eight different genotypes [44,45] and over 100 subtypes [46]. Within a single host, repertoires of viral variants that are highly related but genetically distinct, called "quasispecies", can develop during persistent infection [47]. Mutations around antibody binding sites are crucial in facilitating the selection of transmissible viral variants [48–54], and are aided by the structural flexibility of the virus's CD81 binding site on the envelope E2 protein, which permits different conformations of the protein to bind to the host cell [55,56]. Collectively, these mechanisms create a remarkable capability for mutational immune escape.

HCV also employs a "shielding" mechanism through glycosylation, on the E1 and E2 envelope proteins, which can block the access to conserved epitopes targeted by bnAbs [57–61]. Additionally, HCV can infect cells neighboring an infected one via cell-to-cell junctions, thereby avoiding exposure to neutralizing antibodies altogether [62,63].

Cultivation of HCV in cell cultures has proven to be difficult, likely due to the virus's tropism for highly differentiated hepatocytes. In fact, the first replication of the viral RNA in Huh-7 cells (a line derived from human hepatoma tissue) was not accomplished until 1999, and was only achieved by substituting the structural protein with a gene encoding for neomycin resistance. To facilitate replication of whole virions, it was necessary to introduce adaptive mutations which sometimes also led to the emergence of genetic variants that were not representative of the original clinical isolate [64]. It took further developments and the discovery of the now well researched JFH-1 and JFH-2 lines to establish cell cultures of HCV capable of replication without adaptations [65–67].

A classical approach to vaccine development often uses live attenuated viruses, created by deleting or inactivating virulence factors and/or subsequent passage through cells to introduce safer adaptations. However, the logistics of HCV cultivation and the potential risk of causing disease has greatly limited the utility of such an approach [68] .

HCV pseudoparticles (HCVpp) have helped to alleviate some of these challenges by offering an alternative with which to test subject antiserum at a time when cell culture derived HCV (HCVcc) was not widely available for all genotypes. HCVpp are replication-deficient virus-like particles of various origins that have been engineered to carry HCV envelope proteins (i.e. surface antigens) but not any genetic material. Today, HCV cell culture-produced virions are available for some strains of each of the main genotypes 1 to 6 and these HCVcc are consistently used in research. However, these particles still have some different biochemical characteristics in regard to density and association with lipoproteins compared to particles isolated from infected patients [69].

Animal models for HCV are difficult to produce due to the narrow host range of the virus: humans are the only natural hosts for HCV, with the wild-type virus additionally able to infect chimpanzees and tree shrews in an experimental setting. A suitable model must capture both viable HCV replication and a functioning immune response [70], but at the time of writing no model fulfills both criteria to a satisfactory extent. While chimpanzees have previously been valuable in studying HCV [71], they are no longer used due to ethical and financial constraints. Liver chimeric mice can be created through the transplantation of human primary hepatocytes to study human liver infections and evaluate potential treatments [72–76], but they are mostly unsuitable for vaccine research due to their immune-compromised state. An attempt to solve this problem has been made by developing dually engrafted mouse models, produced by transplanting both human hepatocytes and human immune cells from the same donor into an immunodeficient mouse [77,78]. While providing a somewhat better model of a human-like immune response, these models lack functional B-lymphocytes, hampering the study of antibody responses and vaccine development [79]. A third strategy uses transgenic humanized mice which have been genetically modified to support HCV replication [80]. While there are still significant differences in the course of the disease and the immune response compared to humans, this is currently the most viable small animal model available.

Novel HCV-related hepaciviruses and pegiviruses have also been identified in dogs, horses, bats, rodents, and non-human primates [81], but not all are hepatotropic or mimic the natural course of HCV infection.

The inbred Brown Norway rat model infected with NrHV (novel rodent hepacivirus) exhibits clinical symptoms comparable to human HCV infection and is suitable to study some aspects of the hepacivirus immune response that are analogue to HCV [82].

The evaluation and comparison of novel vaccine candidates in both pre-clinical and clinical settings faces a significant challenge in the lack of internationally standardized assays to assess their efficacy. For example, serum neutralization (SN) assays, used to establish the ability of antibodies in serum from a vaccinated subject to prevent viral infection *in vitro*, are commonplace but vary greatly in implementation. As wild-type HCV replicates poorly *in vitro*, SN assays typically use either HCVcc or HCVpp as a proxy. The use of different assays for evaluating the efficacy of antibodies makes it hard to compare research results between laboratories, necessitating the development of standardized tests [83]. It should also be noted that effective neutralization by antibodies cannot be taken to indicate

clearance: broadly neutralizing antibodies (bnAbs) are found in patients with chronic HCV infection, with studies suggesting the timing of generation of these antibodies may play a more significant role in determining whether the infection resolves spontaneously or becomes chronic.

Clinical trials for HCV vaccines additionally face uncertain study outcomes due to the low predictive power of non-clinical evaluation methods, a lack of universally accepted immunological correlates of protection for HCV, and a number of logistical difficulties in ensuring that samples are representative of high-risk populations. The two HCV vaccine candidates tested in humans to date have both shown limited success [84,85]. High-risk populations like people who inject drugs (PWID) [86], HIV infected individuals, and men who have sex with men (MSM) [87] are primary targets for HCV prevention and in the case of PWID and HIV infected individuals complicating vaccine testing due to differences in immune responses or practical considerations in implementing clinical trials. The identification of correlates of protective immunity would also aid in screening of vaccine candidates in volunteers not at risk for HCV [88].

These challenges in conducting clinical trials are prompting researchers to consider controlled human infection models (CHIMs) as an alternative to field trials for evaluating vaccine efficacy. Controlled infection studies offer benefits like lower cost, faster assessment, and insight into immunological mechanisms of vaccine protection [89]. CHIMs have proven successful in studying diseases like malaria and influenza. However, establishing HCV CHIMs includes hurdles like standardizing approaches and identifying suitable endpoints for efficacy assessment. Developing a successful HCV CHIM will require a good understanding of HCV infection, the host immune response, and the introduction of standard protocols and regulatory standards [89,90].

The Feasibility of a Vaccine

There are three main factors in the current evidence which suggest that an HCV vaccine is feasible: natural immunity, cross-reactive immunity, and conserved epitopes. The immune response during the acute HCV infection phase, especially the maintenance of B and T cell responses, helps control the virus [91–93] and the long-term maintenance of these responses is strongly correlated with the spontaneous resolution of HCV infection in about 30% of infected individuals [94–97]. Natural clearance of one HCV strain generates immunity against heterologous strains [98–103] and a reduction of viremia [104,105]. Finally, HCV's conserved epitopes could be targeted for a broad-spectrum vaccine [106–112]. However, effective clearance is not guaranteed by any singular immune response [113]. Thus, while a vaccine replicating natural immunity could be effective, novel immunity-stimulating platforms should also be explored.

HCV Vaccine Development Considerations and Directions

Hepatitis C vaccine research has seen challenges, with the only vaccine reaching a phase 2 trial, a t-cell targeting candidate, failing to prevent chronic disease in PWID [85]. However, neutralizing antibodies (NAbs) targeting HCV envelope glycoproteins are showing promise and today the scientific community agrees that future vaccines should aim to stimulate both cellular and humoral responses

[114,115]. Being the only targets exposed outside of host cells, E1 and E2 glycoproteins have become the key targets for humoral responses [35,36] and much of current research focuses on inducing neutralizing antibodies (NAbs) against these proteins [116]. Recent discoveries have revealed the E1E2 heterodimer structure [117] and expanded our understanding of immune evasion tactics.

Individuals who clear HCV infection often exhibit rapid NAb and T-cell responses [118], similar to those elicited by successful viral vaccines like poliovirus. Crucially, these antibodies should be cross-reactive due to HCV's variability [119,120]. There is a complex relationship between HCV and the immune system: early antibody appearance is linked to HCV clearance, while late development often results in chronic infection [105,121–124]. Animal experiments confirm the protective function of antibodies, with the monoclonal antibody HCV1 notably reducing viral load in chimpanzees [102]. Further studies demonstrated the efficacy of antibodies AR3A, AR3B, and AR4A which are targeting some of the most conserved E2 regions [52,122], with a study by de Jong et al. showing protection in mice [125].

The Existing HCV Vaccine Strategies, their Vaccine Candidates and Key Research Groups working on them

The Δ123 E2 Candidate by Heidi Drummer and Colleagues

Involved Institutions: Burnet Institute

Key research summary

In 2015, the group showed that the hypervariable regions HVR1, HVR2, and igVR reduce antibodies' ability to bind to the E2661 site [126], which is crucial for E2's interaction with CD81 for cell entry. Alhammad and colleagues introduced a monomeric form of E2 lacking these variable regions, termed "core domain" or E2-Δ123, which stimulated broadly neutralizing responses in guinea pigs [126,127]. In 2020, they improved immunogenicity by developing high molecular weight forms of E2-Δ123 from monomers, although the multimeric E2 protein demonstrated reduced bnAb reactivity. Assembled high molecular weight forms of E2-Δ123 elicited antibodies which competed with CD81 for binding to E2,and neutralizing antibodies were generated towards homologous and heterologous HCV genotypes [128]. They later transitioned two E2-Δ123 prototypes to a Duck Hepatitis B virus platform, leading to a cost-effective, highly immunogenic Δ123 DHBV S VLP in 2022 [128,129].

Future directions, Obstacles and Comments

(From personal communication)

Professor Heidi Drummer, with 24 years of expertise in Hepatitis C and vaccine development, is focused on understanding and enhancing the E2 glycoprotein immunogenicity. The current Δ123 protein candidates show potential, but the high molecular weight multimer approach's low yields

and varied production are hindering scalability. Consequently, the team is investigating alternative strategies, like the virus-like particle formed by an altered duck hepatitis B virus (HBV) S protein carrying E2 envelope proteins (see section: HBV-HCV candidate by Elodie Beaumont and Philippe Roingeard). This approach produces good yields of a particle with high immunogenicity and yields, but its specificity may require optimization. Alternatively, assembly of monomeric forms into high molecular weight forms overcomes manufacturing issues [128].

The team is also exploring the possibility of using yeast for growing an HBV-based candidate. However, they must consider the impact of varied glycosylation patterns on this method's efficacy. They are also considering mRNA based options, an approach which could alleviate manufacturing and safety issues. With Moderna having local facilities and the group's participation in the Moderna mRNA access program, the foundations for such an effort are laid.

Similar to other groups, finding funding for phase 1 trials seems to be the main hurdle, with multiple problems playing into this. For vaccine trials to appeal to pharmaceutical companies, researchers need to reduce the risks associated with HCV trials. It is necessary to address the unpredictability of current animal models, the current lack of understanding of immune responses in previously infected individuals, and the absence of standardized assays for testing vaccine candidates. Drummer and her colleagues, Rob Center and Joey McGregor, believe that a standardized evaluation process to compare and assess vaccine candidates effectively is the most important issue that needs addressing in the field globally. It would boost the confidence of the pharmaceutical industry and funding agencies. The stigma of HCV infection and the misconception that an HCV vaccine is unattainable add to the challenges. Finally, in Australia, there is no obvious at-risk target group for HCV vaccine trials. The high infection rates in prison populations may theoretically make them suitable, but ethical considerations make this option less viable to pursue.

The team supports establishing a Controlled Human Infection Model (CHIM) for HCV to reduce the risks of large-scale studies failing in late phases. Likely, virus isolates obtained prior to successful treatment of an infected individual, obtained at an early stage of their infection should be used, with additional detailed analysis of the E1E2 glycoprotein and its recognition by neutralizing and non-neutralizing antibodies. Meeting these challenges could bring us closer to a successful HCV vaccine.

An Inactivated Whole Virus Candidate by Judith M. Gottwein, Jens Bukh and Colleagues

Involved Institutions: University of Copenhagen (lead), The Scripps Research Institute California

Key Research Summary

In 2014, Mathiesen et al. introduced an innovative method for producing HCVcc under serum-free conditions in Huh7.5 cells [130]. This HCVcc, once UV-inactivated and prepared with the AddaVax™ adjuvant (an MF59 analog), induced a robust response of broadly neutralizing antibodies (bnAbs) superior to that resulting from an Alum + Monophosphoryl lipid A (MPLA) adjuvant,

establishing it as a promising initial candidate for an HCV vaccine centered on deactivated whole virions [131]. Further, high-yield cultures produced HCVcc of genotypes 1a, 1b, 2a, 3a, and 4a, amplifying the exposure of neutralizing antibodies (NAbs) epitopes [132]. While the inactivated whole-virus strategy is still in an early stage, the latest high-yield genotype 1-3 HCV vaccine variant, presenting natively folded envelope proteins, has shown significant improvements in neutralizing epitope exposure (e.g. AR3A and AR4A). The resultant vaccine-induced IgG broadly neutralized all HCV genotypes (1-6), showing superior efficacy compared to IgG from chronically infected patients, and thus presenting a promising basis for further preclinical development of a broad-spectrum HCV vaccine.

HBV-HCV candidate by Elodie Beaumont and Philippe Roingeard

Involved Institutions: Université de Tours

Key Research Summary

Beaumont, Roingeard, and their team pioneered the development of a novel vaccine candidate rooted in the small envelope protein of the Hepatitis B Virus (HBV S) which is capable of self-assembly into virus-like particles (VLPs). They combined full-length genotype 1a HCV envelope glycoproteins (E1 and E2) with the HBV S protein, creating chimeric HBV-HCV proteins (E1-S and E2-S). When these proteins were co-expressed with the wild-type S protein in mammalian CHO cells, they successfully co-assembled into subviral particles (SVPs) that were secreted and exhibited high immunogenicity. Immunization of New Zealand rabbits with these chimeric SVPs triggered a dual humoral response against both HBV and HCV. Crucially, the antibodies generated were capable of neutralizing a broad variety of HCV genotypes [133–135]. In a further step, a cocktail of particles carrying chimeric E2-S proteins from assorted genotypes was found to induce antibodies with superior neutralizing properties compared to antibodies induced by immunization with genotype 1a E1-S + E2-S particles exclusively [136].

More recently, Beaumont, Roingeard, and their team aimed to improve the neutralizing potential of these antibodies by considering the role of the apolipoprotein E (apoE) in the folding of the HCV envelope glycoproteins, and therefore in the modulation of the HCV sensibility to antibody-mediated neutralization. To mimic the epitopes at the apoE-HCV envelope proteins interface, they developed new chimeric HBV-HCV SVPs bearing envelope proteins complexed with apoE. While apoE improved the folding of HCV envelope proteins, its presence reduced the E2-S protein incorporation. Immunization of New Zealand rabbits displayed varied anti-E1/-E2 antibody titers based on the presence or absence of apoE. ApoE in vaccine particles slightly delayed but enhanced the anti-E1 immune responses in immunized rabbits, probably due to improved E1-S protein folding, resulting in greater immunogenicity; however, it led to a quicker decrease in anti-E2 responses, likely due to reduced E2-S protein incorporation in the apoE bearing particles. Nevertheless, the anti-E2 antibodies generated in the presence of apoE were found to have better neutralizing properties than those induced by the prototype S+E2-S SVPs [137].

Future directions, Obstacles and Discussion

(Partially from personal communication)

The team is in the process of creating a B-cell-centric vaccine that employs HBV S as a vehicle for E1 and E2 HCV glycoproteins. They believe the benefits of this approach are twofold: Firstly, the method of utilizing the HBV S backbone has been validated by the Malaria RTS,S vaccine, which has demonstrated encouraging outcomes in clinical trials and exhibited immunogenicity in individuals previously vaccinated against HBV [138]. Secondly, a combined HBV-HCV vaccine, presenting epitopes of both viruses, could potentially integrate more seamlessly into vaccination schedules.

Recent trials integrating HCV glycoproteins complexed with apoE have shown potential. The prototype vaccine candidate was not particularly competitive, but the latest proposed version, which incorporates apoE on the vaccine particles' surface, emerges as a formidable contender in the field of antibody-centric candidates [137]. The differential incorporation and enhanced folding of proteins could potentially boost their detectability and immune response, bringing them closer to the natural E1 and E2 conformations.

However, challenges persist: despite the adequate public funding for foundational research, the team has struggled to secure funding for a phase 1 clinical trial. They firmly believe that the mitigation of risks associated with clinical trials to make them more appealing is crucial. They also think that efforts to establish standardized neutralization assays will significantly help achieve this goal by making results more comparable across trials. The group is currently conducting HCV pseudoparticle (HCVpp) neutralization tests using the antigenically diverse, representative panel of HCV envelope glycoproteins developed by Justin Bailey's group and collaborators. In addition to this valuable tool, an open-source panel of HCV in cell culture (HCVcc), similar to that developed by Thomas Pietschman, would be beneficial. When asked about the potential value of a human challenge model for HCV, Beaumont and Roingeard expressed that it could provide useful information, yet the team believes it is currently most pressing to conduct more phase 1 clinical trials.

A sE2-Ferritin Candidate by Jin Zhong & Zhong Huang and Colleagues

Involved Institutions: Shanghai Institute of Immunity and Infection

Key Research Summary

This vaccination strategy uses Gt 1b TMD-truncated soluble E2 proteins created in *Drosophila* S2 cells as antigens. The different glycosylation patterns on the soluble E2 protein produced by the S2 cells potentially enhance E2's immunogenicity by improving the protein structure's flexibility compared to mammalian cells [139]. This vaccine, when administered to immunocompetent mice, induced antibodies capable of neutralizing genotype 1–7 HCVcc. The hypervariable region 1 (HVR1) was removed from the soluble E2 protein (sE2), but this did not significantly impact immunogenicity.

This vaccine also generated AP33-like and AR3A-like broadly neutralizing antibodies (bnAbs). Furthermore, when combined with Alhydrogel 2% and MPLA adjuvants, the vaccine induced a NAb response and both memory-type and interferon-γ-producing T cell responses in non-human primates

(Rhesus macaques) [140]. The sE2 protein was also evaluated as a cocktail of proteins from 3 genotypes (1b, 1a, and 3a), enhancing cross-neutralizing responses against certain HCVcc compared to the monovalent vaccine [141]. The E2 protein's presentation was also further enhanced by coupling it to ferritin, which self-assembles into nanoparticles [142].

Comments and Potential Future Directions

The most recent research shows that their nanoparticle vaccine variant (multiple sE2 trimers on the surface of a ferritin nanoparticle) is more effective in inducing broader neutralizing responses compared to the group's previous work, which had already demonstrated the potential of sE2 protein to induce bnAbs. By growing and purifying sE2 from drosophila cells, altered glycosylation patterns are believed to have contributed to the increased infection protection in humanized mice [139] and nonhuman primates [140].

In the latest improvement, the authors argue that the sE2-ferritin nanoparticle's enhanced immunogenicity could be due to its surface conformation, which resembles the likely naturally occurring trimeric E2 on the infectious virion [143]. It may also trigger a more efficient immune response than the sE2 subunit vaccine, as nanoparticles are easily captured and processed by antigen-presenting cells (APCs), promoting their maturation and immune response activation [144]. This is backed by findings showing the ability of nanoparticles to reach lymph nodes, present antigens to resident APCs, and stimulate T- and B-cell response [145]. The authors suggest that the next logical step would be to combine the demonstrated enhancement in the potency and breadth of neutralizing antibody (NAb) responses, observed when using the sE2-ferritin nanoparticle as opposed to the sE2 protein alone, with the improvements observed from their trivalent combination of sE2 (non-ferritin) [141]. This amalgamation would lead to a multivalent sE2-ferritin candidate and, if successfully pursued, will hopefully further increase the neutralization efficiency.

A Virus Vectored T-Cell Vaccine (and A Combined Bivalent Strategy) by the Research Groups around Eleanor Barnes, Andrea Cox, Heidi Drummer and Colleagues

Involved Institutions (Selection): University of Oxford (T-cell vaccine development), Johns Hopkins University Baltimore and collaborators (stage 1/2 trial), Burnet Institute (Δ123 component for bivalent vaccine)

Key Research Summary

In 2012, Barnes and colleagues conducted the first human trial of a T cell-targeting vaccine, incorporating the non-structural genes NS3-NS5B as immunogens [146]. This trial tested two replication-defective vectors based on adenoviruses of rare serotypes: Human adenovirus 6 (Ad6) and chimpanzee adenovirus 3 (ChAd3). The vectors demonstrated a good safety profile and were well tolerated, exhibiting promising immunogenicity. Two years later, published results demonstrated that ChAd3 boosted with an Modified Vaccinia Ankara vector generate high levels of both CD8+ and

CD4+ HCV specific T-cells targeting multiple HCV antigens and leading to sustained memory and effector T-cell populations in a heterologous prime-boost vaccination strategy [147].

This prime-boost strategy was also tested in patients with chronic HCV infection of genotype 1 with the vaccinated patients exhibiting weaker T-cell responses [148]. Despite being well-tolerated, the vaccines didn't significantly affect HCV viral load in patients. However, it is worth noting that treating chronic patients, while an important consideration, is not an imperative objective of the vaccine. Trials were extended to patients who had suppressed viral loads and were undergoing treatment with interferon/ribavirin therapy [149]. Again, the vaccine proved to be well-tolerated and succeeded in inducing HCV-specific T-cell responses in a majority of the patients.

In 2018, improvements to the immunogens transported by the viral vectors were trialed. A computer algorithm was utilized to pinpoint stable genomic regions of HCV that spanned various viral subtypes, including the variations in HCV subtypes 1a and 1b, genotypes 1 and 3, as well as genotypes 1-6 [150]. The efficacy of these constructs, vectored through the simian adenovirus ChAdOx, in eliciting an immune response was examined in C57BL/6 mice: the conserved segment successfully stimulated robust HCV-specific T-cell responses, generating IFN-γ and TNF-α. Additionally, these responses were shown to be cross-reactive with several HCV subtype antigens.

To determine the ideal vaccination schedule, in 2020 Capone and colleagues assessed the outcomes of several boosting strategies of ChAd3 and modified vaccinia Ankara (MVA) platforms in a phase 1 clinical trial [151]. The study demonstrated that after an initial Ad/MVA prime-boost vaccination, the most beneficial reboosting time point is after an extended period and can be effectively achieved using only MVA in the booster. Interestingly, a dose of MVA that is ten times lower than the standard amount proved just as effective.

The insights garnered from initial clinical trials and vaccine scheduling explorations were encapsulated in a comprehensive Phase 1/2 trial, using the recombinant chimpanzee adenovirus 3 vector priming vaccination, followed by a recombinant MVA boost [85], alas without the aforementioned reduction in the MVA boosting dose due to results of the 2020 study by Capone et al. not being available in time. Despite its safety and ability to generate HCV-specific T-cell responses, this regimen failed to prevent chronic HCV infection among a high-risk population of PWID. Notably however, the viral load of vaccines during acute infection was lowered and these same vectors had demonstrated a stronger immune response in healthy adults [147] (see above).

Recent Developments: Towards a combined B- and T-cell Vaccine

Only two vaccine strategies have reached the stage of human trials (also see chapter: Genotype 1a E1/E2 Glycoprotein Vaccine and Bivalent Approaches by Houghton and Colleagues below), but both have faced issues with mutational escape and sequence variability, leading to their inability to prevent chronic HCV despite their capability of generating immune responses [85,111]. This suggests that there is a need for alternative vaccine strategies that better target multiple HCV genotypes and generate both neutralizing antibodies and reactive CD4+/CD8+ T cells for greater vaccine efficacy.

In 2022, a collaboration between the groups lead by E. Barnes in Oxford and by H. Drummer at the Burnet Institute compared different vaccine strategies which combined the T-cell approach outlined above with the B-cell HCV vaccine developed at the Burnet Institute (also see chapter: The Δ123 E2 Candidate by Heidi Drummer and Colleagues) to generate immune responses to conserved epitopes [152]. The sequential vaccination with bivalent viral vector vaccines (ChAdOx1 and MVA)

encoding both Gt1-6 and E2Δ123 immunogens (Gt1-6-E2Δ123) generated both cross-genotype T- and B-cell responses. Both groups currently pursue other collaborations and no further testing of this bivalent combination is planned.

The group around E. Barnes now focuses on multiple strategies to eventually select the most potent antigens as well as delivery platforms, as part of a broader collaboration under the NIH U19 research program [153]. One promising antigen selection comes from a recent lead by Frumento et al. which describes unique features of "clade 1 HCV", containing highly conserved epitopes in bNAb-sensitive E1E2 proteins that are more accessible for antibody binding, thereby enhancing B cell selection expressing bNAb germline precursors and promoting further maturation of bNAb-expressing B cell lineages [154]. Continuous exposure to heterologous antigenic clade 1 E1E2 variants exposing the same conserved epitope could boost this process, while simultaneously disfavoring maturation of B cells targeting less conserved epitopes [154]. Selecting antigens from clade 1 E1E2 to target B-cell stimulation and antigens from the conserved epitope sequences mentioned above[150] for T-cell activation could therefore be trialed in various platforms. The group will explore own soluble E2 as well as mRNA and the R21 platforms, the latter of which already proved successful as a base for a Malaria vaccine [155]; By using the same underlying techniques, Nanoparticles could be formed from a single HCV-HCB surface antigen fusion protein without the need for excess HBsAg which promises to increase the proportion and density of HCV antigen on the resulting VLP. Ultimately, the group's aim is to combine the best performing T-cell and B-cell immunogens in the most potent and practical delivery platforms to overcome the unique challenges posed by the Hepatitis C virus.

Genotype 1a E1/E2 Glycoprotein Vaccine and Bivalent Approaches by Houghton and Colleagues

Involved Institutions: University of Alberta, University of Copenhagen, Saint Louis University

Key Research Summary

The E1E2 heterodimer vaccine, developed by the team of the Nobel laureate Dr. Michael Houghton, is a pioneering B-cell vaccine candidate for HCV. The vaccine uses E1 and E2 proteins of the HCV-1 strain (Genotype 1a) purified from mammalian cell cultures (Chinese hamster ovary (CHO) cells). In trials, immunization of chimpanzees with this vaccine triggered strong humoral responses, protecting them from HCV challenges, and only delaying the resolution of acute infection if low antibody titers were elicited [156]. The antibodies induced were found to cross-neutralize HCV in vitro [157] and in a phase 1 clinical trial (NCT00500747), healthy subjects were immunized with the E1E2 heterodimer and MF59 adjuvant. This resulted in the production of antibodies and replication of $CD4^+$ T cells [84] . However, reactive antibodies against HVR1 were found in half the samples, and only about 50% of participants developed antibodies able to cross-neutralize HCVcc carrying HCV envelope proteins from various genotypes. Genotypes 1a, 1b, 2a, 4a, 5a, and 6a were better neutralized than genotypes 2b, 3a, and 7a [111,158,159]. An experiment in which mice were immunized with the vaccine candidate with HRV1 deleted did not yield antibodies with superior

cross-neutralizing properties compared to the wild-type (WT) E1E2 proteins [160]. Upon detailed examination, it was discovered that the isolate-specific neutralization sensitivity is determined by the N-terminal hypervariable region 1 (HVR1) of the E2 protein. The HVR1 of the J6 strain, which is resistant, shows an affinity to the scavenger receptor class-B type-1 (SR-B1). In contrast, the sensitive JFH-1 strain does not exhibit such binding [161]. The team is also working on a T-cell component of synthetic peptides comprising of conserved HCV epitopes that are readily presented by MHC receptors and induce strong T-cell proliferation (from personal communication) to be used in an adjuvanted combined B- and T-cell vaccine approach.

Beyond the Spotlight: A Summary of Additional HCV Vaccine Candidates

Quadrivalent HCV VLP (Victoria)

In a 2016 study, mammalian-cell-derived HCV VLPs incorporating core, E1, and E2 of HCV genotype 1a were biochemically and morphologically characterized, demonstrating the ability to bind to dendritic cells and generate neutralizing antibody responses in BALB/c mice, as well as core-specific CD8+ T-cell responses in humanized MHC class I transgenic mice indicating their potential for the development of an effective HCV preventative vaccine [162]. In 2018, the group expanded the candidate to a quadrivalent genotype 1a/1b/2a/3a HCV VLP vaccine to broaden the immune responses [163]. The results showed neutralizing antibody, and T and B cell responses in vaccinated mice. Selective neutralizing human monoclonal antibodies targeting conserved antigenic domain B and D epitopes of the E2 protein exhibited strong binding to the novel HCV VLPs. In 2019, testing was expanded to a large animal model: intradermal microneedle vaccination of pigs with the quadrivalent HCV VLP vaccine resulted in long-lived multi-genotype specific and neutralizing antibody responses, along with strong T cell responses, without requiring an adjuvant, demonstrating the vaccine's potential for inducing broad immune responses in larger organisms [164]. However, due to a shift of their research focus after the COVID-19 pandemic and an altered funding landscape, the group is no longer pursuing the development of their vaccine candidate (Joe Torresi, personal communication).

Flag-tagged E1E2 (Gdansk)

The envelope glycoproteins E1E2 of Hepatitis C virus are retained in the endoplasmic reticulum (ER) membrane, which makes their purification difficult. To overcome this, Krapchev and colleagues suggested fusing these proteins into molecules to maintain their conformation and facilitate purification. The E1E2 was tagged with a Flag tag, which aided purification without hindering the proteins' folding or heterodimerization, or affecting the CD81 binding site. Serum from mice, immunized with HCV genotype-derived sE1E2-Flag (1a or 2a), underwent a neutralization assay. This assay gauged the antibodies' efficiency in neutralizing HCV1a and HCV2a by inhibiting the infectivity of 50 focus forming units (FFAs, a measure of the number of infectious viral particles) added to the diluted serum, in Huh7-J20 host cells. Approximately 60% of units were prevented from establishing infection [165].

Fc fused E1E2 (Fc-E1E2) Heterodimers (Beijing)

Lin et al. developed another protein-based vaccine utilizing Fc-E1E2 proteins consisting of an E1E2 heterodimer fused with a human IgG1 Fc fragment. Fc is the constant tail region of the antibody which, rather than binding to antigens, interacts with other components of the immune system including complement proteins and cell-surface receptors on multiple immune cells. Fc-E1E2 correctly folds into heterodimers. The group further explores its use as a pentavalent antigen for enhanced performance. This vaccine is designed to induce a broadly neutralizing antibody (bnAb) response against HCV, and targets conserved HCV epitopes at the CD81 binding site of E2, including discontinuous epitopes in antigenic region 3 (AR3) and a continuous epitope in antigenic site 412 (AS412). The pentavalent form of the vaccine stimulates better neutralizing antibody production in mice compared to its monovalent counterpart. However, the structural complexity of the E1E2 heterodimer may impact the durability of the induced neutralizing antibody. Despite this, the Fc-E1E2 vaccine shows promise due to its potential to elicit bnAbs targeting multiple epitopes across various HCV strains [166].

Multi genotype mosaic E1E2 nanoparticle (Amsterdam)

Sliepen and colleagues proposed trimeric permutated E2E1 Gt 1a nanoparticles. The HCV envelope glycoproteins were rearranged to bring the N-terminus of E1 and the C-terminus of E2 closer together to facilitate interactions between E1 and E2 proteins [117]. The immunization of New Zealand female rabbits with these E2E1 nanoparticles led to the development of antibodies able to neutralize more HCVpp than a monomeric E2 protein. Additionally, they developed E2E1 mosaic nanoparticles (incorporating E2E1 from multiple genotypes). These nanoparticles induced slightly better cross-neutralizing antibodies than a cocktail of 6 E2E1 nanoparticles each containing proteins from a single strain [167].

Inactivated HCVcc (Tokyo, Kanagawa, Kyoto)

An UV-inactivated HCV vaccine, developed by Akazawa and colleagues, was based on a genetically engineered replication-deficient HCV based on Gt 2a [168,169]. These particles were produced in large-scale Huh7.5.1 cell cultures and then purified, with a recovery rate of 15% [170]. When BALB/c mice and marmosets (nonhuman primates) were immunized with this inactivated HCVcc, they produced cellular responses and antibodies against HCV structural proteins that were able to neutralize HCV pseudoparticles (HCVpp) and HCVcc of different genotypes [169,170]. One advantage observed with this vaccine was that lower doses were needed to induce antibody production compared to a recombinant protein. Moreover, antibodies induced by the immunization of human liver chimeric mice protected these animals from HCV challenge.

Soluble E1E2 Prime-Boost (La Jolla, Scripps Research Institute)

Another antigen developed at the Scripps Research Institute consists of a novel soluble E1E2 heterodimer (sE1E2) with the transmembrane domain removed, and varying combinations of linkers and Strep-Tag 2 inserted between the E1 and E2 ectodomains. When delivered to mice using a DNA-prime/protein-boost strategy, sE1E2 yielded improved antibody titers and reactivity when compared to the wild-type cell-associated E1E2. Despite a weak neutralizing effect, serum antibodies

mainly targeted specific E1 and E2 regions, rather than the typical E2 hypervariable region. Some monoclonal antibodies generated were able to cross-neutralize multiple HCV isolates, indicating that sE1E2 can prime B cells for universal neutralizing epitopes, despite producing an inadequate serum neutralizing antibody response. This could provide a promising blueprint for rational antigen engineering. The epitopes targeted by the polyclonal antibody responses were identified, and monoclonal antibodies were generated. Antibodies primarily targeted E1 region 211-250 and E2 regions 421-469, 512-539, 568-609, and 638-651, rather than the well-known immunodominant E2 hypervariable region 1 (HVR1). Surprisingly, around 12% of isolated monoclonal antibodies were specific to the conserved E2 antigenic site 412-423, and 85% of these could cross-neutralize multiple HCV isolates. The epitopes recognized by these antibodies have similarities but are distinct from the previously reported HCV1 and AP33 broadly neutralizing epitopes [171].

Ferritin Nanoparticle E2 Core (La Jolla, Scripps Research Institute)

In a recent study by He et al., self-assembling E2 core nanoparticles were developed using a derivative of H77 (Gt1a) and HK6a (Gt2a). The team employed an ensemble-based de novo design to create a truncated variant of the Variable Region 2 (tVR2). Initially, the design was based on H77, but the most successful design was eventually extended to HK6a (GT6a).

These novel E2 "mini" core variants, or E2mc3, demonstrated enhanced thermal stability and antigenicity compared to the E2 core constructs previously developed by the same group (Kong et al. 2013). The past research by Kong and colleagues had already demonstrated the structural flexibility of the E2 CD81 receptor-binding site, explaining how this flexibility could potentially facilitate the promotion of non-neutralizing antibodies over neutralizing ones.

He et al. displayed the selected E2mc3 variants on different nanoparticle platforms: ferritin (24-mer), E2p (60-mer), and I3-01(60-mer). This further enhanced antigenicity when compared to individual E2, and induced high titers of antibodies. Interestingly, the NAb response varied significantly, with epitope mapping revealing a more "effective" NAb response to the Front Layer (FL) and Antigenic Site 412 (AS412) for the E2mc3-ferritin variants [172].

Synthetic Consensus E2 Glycoprotein (Nottingham)

Tarr et al. attempted to minimize the genetic diversity between the vaccine and circulating HCV strains. They developed two synthetic E2 glycoprotein immunogens, NotC1 and NotC2, based on consensus nucleotide sequences from circulating genotype 1 HCV strains. These were recognized by broadly neutralizing monoclonal antibodies. Guinea pigs immunized with these immunogens developed high titers of cross-reactive anti-E2 antibodies. While the antibodies could neutralize some genotype 1 viruses, they were less effective against genotypes 2 and 3. The study concludes that, for a successful vaccine, a more focused approach directed at common epitopes is needed to elicit broadly neutralizing antibodies [173].

Prime-Boost Viral-Vectored Gag Protein HCV VLPs (Lyon)

Group-specific antigen (gag) is a precursor protein found in retroviruses which facilitates the creation of virus-like particles (VLPs) resembling enveloped viruses; these are said to be “pseudotyped” as they display envelope proteins from multiple viruses. Garrone et al. created VLPs

expressing the Gag protein of Moloney murine leukemia virus (MLV) as well as the E1 and E2 envelope proteins of HCV [174]. These VLPs, which lack a viral genome or enzymes, were generated via vector expression in mammalian cells. Initial pseudotyping attempts with unmodified E1 were unsuccessful, leading to the creation of a modified E1 (E1G) that improved pseudotyping efficiency. Various recombinant vectors were also developed for prime-boost experiments.

In mice, multiple VLP injections were required to induce antibody responses against HCV envelope proteins. However, heterologous prime-boost experiments enhanced immunogenicity, particularly with VLP-E1G. In macaques, a prime-boost regimen with E1 immunogens was tested. While initial results were minimal, boosting with VLP-E1G elicited anti-E1 IgG responses. A second regimen induced T cell responses against E1 and E2, and induced anti-E2 antibodies. Boosting with VLP-E1G and VLP-E1E2 triggered anti-E1 antibodies and boosted anti-E2 antibodies. Post-boost, higher neutralizing antibody titers were detected in all test subjects, showing some cross neutralization capability.

Viral Vectored Measles E1 and E2 (Minnesota)

This team constructed two vaccine candidates based on attenuated measles virus (MV) as a vector. Their vaccine candidates are based on a plasmid identical to the Moraten/Schwartz vaccine strain genomes [175]. They inserted the HCV CE1E2 sequence to create MV-CE1E2 and transferred sequences encoding E1 and E2 ectodomains fused with the MV F protein sequence to construct MV-E1/Ft-E2/Ft. The MV F protein sequence facilitated the transport of E1 and E2 glycoproteins to the cell surface.

Experiments showed that the E1 and E2 proteins were detected on the cell surface, indicating successful transport. Mice tested with virus variants demonstrated neutralization titers and reactivity, with boosted reactivity following a single dose of soluble HCV E2 protein. MV-CE1E2-inoculated mice displayed 90% HCV-specific neutralization, while MV-E1/Ft-E2/Ft-inoculated mice exhibited 70% neutralization. The study concluded that a MV vector expressing unmodified E1E2 glycoproteins can generate cross-neutralizing HCV antibodies in vivo, suggesting the potential efficacy of a prime-boost strategy with vectored MV for developing HCV-neutralizing antibodies.

sHBsAg VLPs carrying E2 residue 412-425, 434-446, 502-520, and 523-535 (Gdansk)

Czarnota and colleagues engineered target specific amino acid (aa) sequences or epitopes which are critical for sparking the creation of neutralizing antibodies (NAbs). Their vaccine is based on the small surface antigen of the Hepatitis B virus, (sHBsAg) which naturally creates virus-like particles (VLPs). In the context of this vaccine, its hydrophilic loops are substituted with epitopes I, II, or III from an HCV E2 protein of genotype (Gt) 1a [176,177].

The resulting hybrid particles were generated using *Leishmania tarentolae*, an expression system that mimics the glycosylation pattern found in mammalian cells. Studies revealed that antibodies directed at epitope I (aa 412–423), which were obtained from vaccinated mice, demonstrated enhanced cross-neutralization abilities against a range of HCV genotypes (1a, 1b, 2a, 2b, 4a, and 5a) in comparison to antibodies directed at epitopes II and III [177].

Conserved Epitope E1, E2, NS4B, NS5A and NS5B peptide strategy (Cairo)

Dawood et al. selected peptides from Gt 4a HCV E1, E2, NS4B, NS5A, and NS5B proteins. These peptides contained residues highly conserved across HCV genotypes and were associated with strong B and T cell responses in spontaneous clearers [178]. Mice immunized with these peptides demonstrated cellular and humoral responses (neutralization of Gt 2a and 4a HCV) in a dose-dependent manner. In another peptide approach by Mosa and colleagues, two peptides from the HVR1 region of HCV exhibiting high sequence divergence were tested in mice. The combined vaccination of these two peptides yielded better immune responses and led to the cross-neutralization of HCVpp. Notably, genotypes 1a, 1b, and 6a were neutralized more effectively than genotypes 2a, 3a, and 5a [179].

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