Editorial on "Broadly neutralizing antibodies abrogate established hepatitis C virus infection" published in *Science Translational Medicine* on 17th September 2014

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Abstract: Hepatitis C virus (HCV) is a blood borne pathogen that causes chronic liver disease and afflicts 170 million people world-wide. While direct acting antivirals now provide a highly effective means to cure those infected with HCV, there is no vaccine to prevent infection. Published in *Science Translational Medicine*, de Jong *et al*. [2014] show that highly potent neutralizing antibodies (NAbs) directed to one of the surface glycoproteins of HCV, E2, can not only prevent infection but can also eliminate established infection in experimental animal models of HCV. They provide compelling evidence that for HCV to maintain a chronic infection, it must infect new hepatocytes; infection cannot be sustained in reservoirs of infected cells alone and that E2-specific NAbs are sufficient to cure an infection. In addition, the manuscript further supports the importance of NAbs in preventing, controlling and possibly curing HCV. Thus NAbs are not only essential to the development of prophylactic vaccines but may yet have a role in therapeutic approaches to HCV treatment.

Keywords: Hepatitis C virus (HCV); neutralizing antibodies (NAbs); vaccine

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Hepatitis C virus (HCV) is now the leading indicator of liver transplantation in most developed countries and deaths due to HCV exceed those caused by HIV in the United States of America. One of the major difficulties for developing antiviral therapies and vaccines for HCV is its high mutation rate of 2.5×10^{-5} per nucleotide per genome replication (1) and its tolerance for such mutations whilst maintaining the ability to replicate. As a result, HCV is classified into seven geographically distinct genotypes that differ by up to 30% at the nucleotide level, and over 60 subtypes that differ by up to 20% at the nucleotide level. Until 2011, the standard of care for HCV was pegylated interferon and ribavirin for 24 or 48 weeks and sustained virological response (SVR) rates ranged from 20-80% depending on infecting genotype, host genetic factors and disease stage. Since 2011, direct acting antiviral agents (DAAs) targeting the HCV proteins have been introduced. On October 10 2014, the FDA approved the first all oral, once a day, interferon and ribavirin free therapy with a SVR rate of >90% following a 12-week course of therapy in treatment naive genotype 1 infected patients. In the future, pan-genotypic therapies will become available providing a means to treat all HCV infections, subject to affordability.

Approximately 70% of those infected with HCV establish a life-long chronic infection while 30% clear their acute infection. The reasons why only some people can spontaneously clear HCV are not completely understood but clearance is at least in part attributed to the quality and specificity of the T cell response. With recent advances in the culture of HCV, new animal models of HCV infection and the isolation and characterization of neutralizing antibodies (NAbs) isolated from HCV infected people, NAbs are now believed to be a key player in control and clearance of HCV and there is growing body of evidence to support their importance.

On the surface of HCV are two viral glycoproteins, E1

and E2 that together initiate the viral replication cycle in liver cells. Glycoprotein E2 is the major surface protein and contains binding sites for cellular receptors and is a major target for NAbs. The first step of HCV infection includes binding to heparin sulfate proteoglycans followed by interactions with scavenger receptor class B type 1 (SR-B1), CD81, claudin-1 and occludin. In addition, several host cell factors have been identified that support HCV entry including epidermal growth factor receptor, ephrin receptor A2 and Niemann-Pick C1-like 1 cholesterol adsorption receptor [for review see (2)]. A requirement of HCV entry is its internalization into endosomal compartments where low pH is believed to drive fusion of viral and endosomal membranes resulting in delivery of the nucleocapsid into the cytoplasm (3).

Critical to HCV entry is the interaction between glycoprotein E2 and the tetraspanin CD81 (4). As CD81 is found on all nucleated cells it does not define the tropism of HCV. Within CD81 is a small and large extracellular loop (LEL) and it is through the LEL that E2 binds at nanomolar affinity (5). The CD81 binding site on E2 is localized within the receptor-binding domain (RBD) spanning residues 384-661 (H77c polyprotein numbering). Four discontinuous sequences form the CD81 binding site with major contributions by amino acids located within residues 420-421, 436-441 and 527-535 (6-8). Consistent with its essential role in attachment of virions to host cells, antibodies that interfere with this binding event are generally neutralizing and prevent HCV infection *in vitro* and *in vivo* (9).

While cell-free virions appear to be highly susceptible to antibody-mediated neutralization, an alternative pathway of HCV transmission involving cell-cell spread may be more resistant to the effects of NAbs (10-12). Cell-cell transmission of HCV is actin-dependent and requires the involvement of CD81, SR-B1, claudin-1 and occludin and occurs more rapidly than cell-free HCV infection (12,13). In addition, recent studies have shown that transmission of drug-resistant HCV variants also occurs primarily through cell-cell transmission (14). Studies employing both human and rodent derived NAbs specific to E2 reveal that cell-cell transmission occurs in their presence and suggested that once infection of liver cells is established, clearance by NAb alone would be difficult to achieve (10,11). However, one study using an E2 specific broadly neutralizing alpaca nanobody was able to block cell-cell transmission (15). As such, it remains unclear whether cell-cell transmission is indeed resistant to the effects of all NAbs and its relative importance *in vivo* in sustaining HCV infection.

In the paper of de Jong *et al*. published in *Science Translational Medicine*, new evidence is presented that NAbs specific to E2 have the capacity to not only prevent HCV infection of liver cells, but can also clear an established infection (16). These data suggest that cell-cell transmission may not be a dominant route for HCV to infect new cells or that some NAbs can indeed block this route of transmission. In the study, the authors focus on three highly potent cross-neutralizing monoclonal antibodies (MAbs) isolated from chronically infected patients; AR3A, AR3B and AR4A. Both the AR3A and AR3B epitopes are located within the E2 RBD in antigenic region 3 and include contact residues within the 523-540 region, inhibit the E2-CD81 interaction and neutralize all six major genotypes. By contrast, AR4A is specific to antigenic region 4 and only recognizes an E1E2 complex. The epitope of AR4A includes residues from within E1 as well as E2 residues R657, D658, D698 and L692. Both D698 and L692 are located within the membrane proximal external region of E2 involved in heterodimerization with E1 (17) and AR4A does not inhibit the E2-CD81 interaction. Despite this, AR4A neutralizes all six major genotypes. Each of AR3A, AR3B and AR4A were previously demonstrated to protect against HCV challenge in mouse models of infection (18,19).

The authors examined the *in vivo* protective efficacy of AR3A, AR3B and AR4A. Recombinant adeno-associated viruses (AAV) encoding each of the antibodies were administered into mice through intramuscular vaccination. High levels of circulating IgG were detected in serum for at least 16 weeks in both immunocompromised non-obese diabetic $Rag1^{-/-}$ $IL2R\gamma c^{null}$ (NRG) mice and immunocompetent FVB mice. Serum obtained from vaccinated FVB mice neutralized all HCV genotypes examined (1a, 1b, 2a, 4a, 5a and 7a) using an *in vitro* assay of HCV neutralization. To study whether circulating anti-E2 IgG could protect against HCV challenge *in vivo*, Rosa-26 Fluc mice were vaccinated with AAVs expressing the MAbs and their livers transduced with adenoviruses encoding each of the essential HCV entry factors, SR-B1, CD81, claudin-1 and occludin to enable HCV infection. Mice were then challenged with cell culture derived HCV expressing the Cre recombinase to induce luciferase expression. Each of the MAbs individually, and all three in combination, protected mice against HCV challenge. In addition, highly engrafted human liver FNRG mice were injected with AAV vectors encoding the three MAbs and 16 days later challenged with a low dose of genotype 1a HCV. All three vaccinated/challenged animals were protected from HCV

infection, whereas 2/3 animals vaccinated with control AAV became viraemic. Protection of highly engrafted human liver chimeric FNRG mice from HCV infection was also achieved by passive transfer of a cocktail of the three purified MAbs, verifying that protection was due to neutralization by IgG and not a result of AAV infection per se.

The protective efficacy of AR3A, AR3B and AR4A was further studied in primary human fetal liver hepatocyte cultures (HFLC), which support prolonged HCV replication, are non-proliferating and have intact innate immune signaling pathways. Infection of HFLC by genotype 2a HCV was prevented by 1 μg/mL of individual purified MAb and by a cocktail of all three MAbs, when added prior to inoculation. Moreover, the MAbs, either individually or in combination, could severely restrict an established infection of HFLC when added 3-day postinfection. Finally, the ability of the MAbs to clear an established infection *in vivo*, was determined in highly engrafted human liver FNRG mice, which were infected with genotype 2a HCV for 17-25 days, followed by passive administration of a cocktail of purified AR3A, AR3B and AR4A IgG every 3 days. The results showed that HCV titres rapidly fell below the limit of detection as a result of the combination of three MAbs. Further, there was no rebound when IgG levels fell below the limit of detection suggesting that the virus had indeed been cleared. By contrast, while MAb cocktail treatment induced a period of viral suppression in genotype 1a HCV-infected mice, the virus later reemerged indicating that the antibodies failed to cure genotype 1a infection, but was not as a result of viral escape. These results suggest that the efficacy of the MAbs in curing HCV infection may be genotype dependent.

The findings presented by de Jong *et al*. suggest that to sustain infection, HCV must invade new cells. The presence of NAbs directed towards E2 is likely to prevent HCV from invading new cells by blocking cell-free virus transmission and viral clearance of infected cells occurs possibly via innate immune pathways and/or cytopathic effects. The author's suggest that cell-cell transmission of HCV may be less important in sustaining infection than first thought. However, the contribution of cell-cell transmission in viral dissemination in the models used here was not ascertained, and it is also not known whether these MAbs were able to prevent cell-cell transmission of HCV. Further experiments are required to examine whether AR3A/AR3B/AR4A can block cell-cell transmission of HCV in established *in vitro* model systems.

It is also possible that the *in vivo* systems used by de Jong *et al*. are more sensitive to the effects of NAbs in clearing infection than in humans and as such may be overestimating their potential clinical benefit. Previous clinical evaluation of a single neutralizing E2 specific MAb in the treatment of HCV showed a 10-fold reduction in HCV RNA levels in 8/25 patients during the treatment period of 4 weeks (20) suggesting that HCV infection of human liver is indeed more resistant to the effects of NAbs than the experimental systems used by de Jong *et al*. How effective AR3A/3B/4A is to treatment of HCV can only be ascertained through clinical evaluation in chronically infected people.

Another important consideration in curing HCV infection in the experimental systems used here is the affinity and/or specificity of the antibody. In the study by de Jong *et al*. [2014], two distinct specificities of antibody were used; two were directed to antigenic region 3 that prevent E2-CD81 interaction, and one to antigenic region 4 that does not prevent E2-CD81 interaction. Experiments performed with a combination of MAbs in HFLCs did not show an obvious synergistic effect over the use of each of the antibodies individually suggesting that MAbs to either of antigenic region 3 or 4 are equally effective at eliminating an established infection. The advantage of using multiple antibody specificities concurrently in therapeutic regimens is that it limits the ability of the virus to escape the effects of NAbs and will be an important consideration for clinical evaluation.

The findings of de Jong *et al*. also provide great hope to the development of vaccines for HCV. Until relatively recently, the requirement for developing a NAb response through vaccination was not believed to be essential for protection or clearance of HCV. Now, with the discovery of several broadly NAbs isolated from HCV infected people, and demonstration of their ability to prevent and cure HCV infection, a vaccine for HCV must be able to elicit such antibody specificities. A key challenge remains in that it appears to be difficult to elicit broadly NAb specificities using conventional virus or protein based immunogens of the glycoproteins as a result of glycan shielding, dominance of hypervariable region 1 in eliciting type specific immune responses and other immune evasion mechanisms (9). Direct acting antivirals will no doubt enable cure of those already infected with HCV, but to ultimately control and potentially eradicate HCV, development of a prophylactic vaccine must remain a high priority.

While the authors did not determine the minimum effective concentration of the antibody cocktail required to eliminate HCV or whether the findings can be extended to other HCV genotypes, the data provide exciting new evidence that NAbs are effective at controlling and indeed

curing established HCV infection and may have therapeutic potential. Given that genotype dependent differences in progression to chronicity also exist, it will be important to further study the capacity of a wider range of human MAbs for their ability to cure infection with other HCV genotypes in order to identify the best combinations of antibodies for therapeutic use. In the era of DAAs to treat HCV, will we need such therapeutic options? The answer will come as DAA use increases and true SVR rates obtained in treatment of chronically infected patients begin to emerge. Those infected with HCV who fail DAA therapy could potentially turn to neutralizing MAbs for treatment. Ultimately, the efficacy of this cocktail of antibodies in curing chronic HCV infection in humans can only be determined in clinical trials.

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