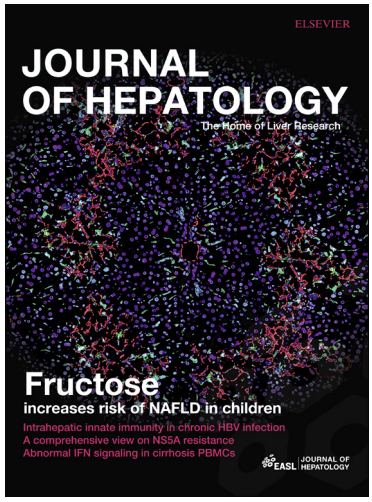

Research Article

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Cross-genotype AR3-specific neutralizing antibodies confer long-term protection in injecting drug users after HCV clearance

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S.J.M, C.B., D. V. B., A.Q.B., H.S. and T.B. are employees of AIMM Therapeutics and S.J.M, A.Q.B., H.S. and T.B. shareholders of AIMM Therapeutics. M.D.J. was a member of the AIMM scientific advisory board.

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Abstract

Background & Aims: Understanding immune protection against hepatitis C virus (HCV) infection is necessary for designing an effective vaccine. A number of broadly-reactive, neutralizing antibodies have been isolated from B cells of HCV-infected subjects. However, it remains unclear whether B cells producing such antibodies contribute to the clearance and long-term immune protection against HCV.

Methods: We analysed the B-cell repertoire of thirteen participants from the Amsterdam Cohort Study among injecting drug users with a median follow-up of 17.5 years. Five subjects ultimately became chronically infected either after primary infection or after reinfection. Eight subjects, at the end of study follow-up, were HCV RNA negative following spontaneous clearance of one or multiple infections. From each subject, 10,000 CD27+IgG+ B cells, collected 0.75 year after HCV infection, were cultured to characterize the antibody repertoire.

Results: Using a multiplex flow cytometry-based assay to study the antibody binding to E1E2 from genotype 1 to 6, we found that a high frequency of cross-genotype antibodies was associated with spontaneous clearance of one or multiple infections (p-value=0.03). Epitope specificity of these cross-genotype antibodies was determined by alanine mutant scanning in four subjects, who were HCV RNA negative following spontaneous clearance of one or multiple infections. Interestingly, the cross-genotype antibodies were mainly AR3-specific and showed cross-neutralizing activity against HCV. In addition to AR3

antibodies, three subjects developed antibodies recognizing AR4 of which one monoclonal antibody showed cross-neutralizing capacity.

Conclusions: Together, these data suggest that a strong B-cell response producing cross-genotype and neutralizing antibodies, especially targeting AR3, contribute to HCV clearance and long-term immune protection against HCV.

Lay summary

Although effective treatments against hepatitis C virus (HCV) are available, 500,000 people die from liver disease caused by HCV each year and approximately 1.75 million people are newly infected which could be prevented by a vaccine.

For the design of such vaccine, more insight in the role of antibodies in the protection against HCV infection is needed.

In a cohort of injecting drug users, we found that antibodies interfering with virus cell entry and recognizing multiple HCV genotypes, conferred long term protection against developing chronic HCV infection.

Introduction

Hepatitis C virus (HCV) is one of the major global public health problems, with worldwide 71 million chronically infected people, which results in 350,000 to 500,000 liver-related deaths per year [1]. Current 'direct-acting antiviral' (DAA) treatment is very effective in clearing infection [2]. However, despite high DAA efficacy, treatment alone is unlikely to eliminate HCV by the year 2030 as envisioned by the WHO [1], since treated subjects may become re-infected if exposure continues. Moreover, worldwide, most of the HCV-infected subjects are unaware of their HCV status because of the prolonged asymptomatic nature of HCV infection and limited access to diagnostic tests [3]. In addition, as a result of the high cost of treatment, a large proportion of the HCV-infected subjects are left untreated [3]. Therefore, for the global elimination of HCV, a preventive vaccine is urgently needed.

Development of a protective vaccine against HCV seems feasible, since spontaneous clearance of the virus without antiviral therapy occurs in 25 to 40% of individuals after primary infection [4] and in 30 to 60% of those who are reinfected following clearance of a primary infection [5]. While clearance of HCV infection has been associated with strong and broad T cell responses [6,7], little is known about the role of antibodies in spontaneous clearance of HCV infection and protection upon re-exposure.

HCV clearance has been associated with a neutralizing antibody response against HCV E1E2 glycoproteins during the early phase of infection [8,9]. In addition, a number of broadly neutralizing antibodies have been isolated from B

cells of chronically infected subjects. In general, they target one of three epitopes which are located in or around the CD81 binding site: epitope I (amino acids 412-423), epitope II (amino acids 434-446) and domain B (amino acids 523-535) [10-12]. Interestingly, broadly neutralizing antibodies targeting epitope I or epitope II (AP33, HC84.26) protected humanized mice against HCV genotype 1b infection [13,14]. It has also been shown that AR3A and AR3B antibodies targeting both epitope II and domain B, jointly referred to as antigenic region 3 (AR3), protected mice against HCV infection from isolate H77 (genotype 1a) and isolate J6 (genotype 2a) in combination with AR4A, an antibody recognizing antigenic region 4 (AR4) [15-17]. It remains, however, unanswered whether such broadly neutralizing antibodies contribute to spontaneous clearance and long-term immune protection in humans with ongoing exposure.

The Amsterdam Cohort Study (ACS) among drug users (DU), is a prospective cohort study investigating HIV and HCV infections with regular collection of serum, peripheral blood mononuclear cells (PBMC), and socio-demographic and risk behavioural data [18]. The ACS-DU cohort started in 1985 and came to an end in 2016 which makes it a unique cohort with one of the longest follow-up of DU worldwide [19]. Importantly, all ACS-participants who are injecting drug users (IDU) are, most likely, frequently (re-)exposed to HCV, since 65% reported at least weekly injecting drug use at enrolment in the cohort, with a baseline HCV antibody prevalence of 82% and an incidence of up to 28 primary infections per 100 person-years among IDU [20]. Such a well-characterized cohort of highly exposed subjects with a long follow-up presents a unique opportunity to study the

role of broadly neutralizing antibodies in the spontaneous clearance of HCV infection and protection upon re-exposure.

Here, we studied the frequency, cross-reactivity and epitopes of HCV E1E2 specific antibodies produced by memory B cells from ACS participants. We found that the frequency of HCV specific cross-genotype antibodies, targeting mainly the antigenic region 3 (AR3) and antigenic region 4 (AR4), was associated with spontaneous clearance of one or multiple HCV infection(s) and absence of HCV RNA at the end of the study period.

Material and methods

Subjects

Subjects were selected from the ACS-IDU. After signing a written informed consent, ACS participants visited the Amsterdam Health Service every 4 to 6 months, where blood was drawn for laboratory testing, PBMCs were stored and where they completed a standardized questionnaire on risk behaviour [18]. Our study relies in part on self-reports, which have been shown to be a valid method for measuring behavioural variables in our cohort, where social desirability does not play an important role [21].

For the study presented here, out of 106 subjects with an acute or recent HCV infection we selected those with at least 5-year follow-up after primary infection with available PBMCs. Acute primary infection was defined by seroconversion for HCV antibodies (AxSym HCV version 3.0; Abbott) during follow up [22]. Recent infection was assumed when at cohort entry HCV antibodies were already

present in subjects who had started injecting drugs less than two years before. We excluded subjects who were HIV-seropositive or positive for HBV surface antigen (AxSym HBsAg, Abbott) at the time of HCV infection.

For seroconverters, the date of the infection was estimated by calculating the midpoint between the last HCV seronegative visit and the first HCV seropositive visit. The date of infection for the recent infections was estimated as the midpoint between the start of injecting drugs and cohort entry [22].

HCV viremia was determined for these subjects at yearly intervals using a transcription-mediated amplification assay with a detection limit of 5 IU/ml (Versant; Siemens Medical Solutions Diagnostics). If HCV RNA was detected, genotyping was performed by sequencing the NS5B region [23]. Clearance was defined as being HCV RNA negative at two subsequent time points. After clearance, in some subjects, reinfection was documented, as evidenced by at least one HCV RNA positive time point during follow-up following clearance of an earlier infection. Chronic infection was defined as continuous viremia for at least two years after the estimated date of (re)infection. This study was approved by the Medical Ethical Committee of the AMC and informed consent was obtained from all subjects. HCV genotype, HBV status and HIV status are summarized in Supplementary Table 1.

B cell isolation and cultures

From ACS participants, frozen PBMC were obtained and CD27+IgG+ B cells were immortalized as described previously [24]. In brief, B cells were isolated

and, after stimulation, the cells were transduced with a retroviral vector containing the transgenes BCL6, BCL-xL and green fluorescent protein as marker gene. Transduced B cells were maintained in culture with gamma-irradiated CD40 Ligand expressing L-cells and recombinant mouse interleukin-21. The transduced B cells secrete immunoglobulins (antibodies) into the culture supernatant.

Multiplex flow cytometry assay

To determine the breadth of the antibodies, we performed a multiplex flow cytometry assay as described previously [25]. In brief, antibodies present in B cell supernatants were incubated with a mix of fluorescently barcoded 293T/17 cells (ATCC) transfected with full-length E1E2 derived from six HCV genotypes (Supplementary material and methods). After addition of the secondary polyclonal goat anti-human IgG-PE antibody (Southern Biotech), the fluorescence was measured using a FACSCanto™ II flow cytometer (BD Biosciences). To determine E1E2 antibody binding, the value obtained for binding to E1E2 transfected cells was subtracted from the value obtained for binding to non-transfected cells and normalized by using the average of the seven negative antibody controls. The cut-off value for binding was based on values of 882 B cell supernatants from three HCV uninfected subjects (Supplementary Fig. 1, Supplementary material and methods).

Enzyme-linked immunosorbent assay (ELISA)

To determine the binding characteristics of the antibodies, ELISA assays were performed as described previously [26]. In brief, plates were coated either with the HIS-tagged E2 ectodomain (Supplementary material and methods) or with *Galanthus nivalis* lectin on which E1E2 protein containing 293T/17 cell lysate was captured. Then, antibodies present in B cell supernatant were added and binding was detected by horseradish peroxidase-conjugated polyclonal goat anti-human IgG (Jackson). Optical density (OD) at 450nm was measured with an EnVision Multilabel Reader (PerkinElmer).

HCV pseudoparticle (pp) neutralization

Neutralization activity of antibodies was determined using the HCVpp system based on Luciferase reporter gene activity (Supplementary material and methods).

HCV cell culture (cc) neutralization

Neutralization activity of antibodies was determined using the HCVcc system as described previously [27,28]. The detailed procedure is described in the supplementary material and methods.

Antibody sequencing

The variable regions of the antibody heavy and light chain from the cross-genotype B cells were sequenced as described previously [29]. In brief, total RNA was isolated from B cells and after generation of cDNA, the cDNA from the

heavy and light chain were specifically amplified by polymerase chain reaction. Finally, the sequences were obtained by Sänger sequencing using BDT reagents (Thermo Fisher Scientific) and were submitted to the IMGT/V-QUEST database to assess V(D)J usage and somatic hypermutation status [30].

Data analysis

Data were analysed using Prism software (GraphPad). Statistical comparison between categories was performed using an one-tailed Mann–Whitney test, as we hypothesized that cross-genotype antibodies were more frequently present in subjects who spontaneously cleared infection(s) (software package SPSS, v. 19.09, IBM). Flow cytometry data were analysed using FlowJo software (FlowJo).

Results

Characteristics of the study population.

Thirteen cohort participants with acute or recent HCV infection met the inclusion criteria for this study. The median age of the selected subjects was 29 years (interquartile range (IQR) 23 - 33) at the time of HCV infection (Table 1). The median follow-up time of these subjects since primary HCV infection was 17.5 years (IQR 11.7 - 18.9). The subjects were classified into two groups based on the outcome of HCV infection:

(i) subjects who became chronically infected (CHRs; n = 5) either after primary infection (CHR1-3), or after HCV reinfection (CHR 4-5);

(ii) subjects who cleared one or more infections and were HCV RNA negative at the end of follow-up (CL1-8). All subjects who cleared the infection were presumably re-exposed to HCV as they continued injecting drugs for a median of 5.9 years after primary infection during a time period (1986-1994) of extremely high HCV incidence (10 - 28 per 100 person-years) in this cohort [20]. Frequent re-exposure was also evidenced by the occurrence of one or more reinfections, which were documented in eight of thirteen subjects. Characteristics of all thirteen subjects are summarized in Table 1.

High frequencies of cross-genotype antibodies in CLs

From each subject, 7 to 15 millions PBMC were available that had been collected 0.75 year (IQR 0.54 – 1.12) after HCV infection (Table 1). From these PBMC, CD27+IgG+ B cells were purified and subsequently immortalized [24]. A total of 10,290 cells per subjects were cultured as mini-cultures at a density of 35 B cells per well. Antibodies secreted in the B cell supernatant were analysed for binding to 293T cells transfected with full-length E1E2 from HCV genotypes 1a, 2b, 3a, 4, 5 and 6, using the multiplexed flow cytometry assay as described before [25]. At a density of 35 cells per well, the percentage of positive wells was below or equal to 37%. We, therefore, assumed based on the Poisson's distribution, that only one out of the 35 cells produced E1E2 specific antibodies. The median frequency of E1E2 specific antibodies in the B-cell repertoire was 14.6 antibodies per 10,000 CD27+IgG+ B cells for CHRs (IQR 3.9 - 18.5) and 16.5 antibodies per 10,000 CD27+IgG+ B cells for CLs (IQR 3.6 - 77) (Fig. 1A). Although the

frequencies did not differ between the groups, the antibodies from CHRs were mainly genotype-specific and directed against the genotype of the ongoing infection. This confirmed findings by Tarr et al., who described the relationship between serum antibody reactivity and genotype of infection [31]. Antibodies from CLs showed a much broader reactivity as compared to CHR derived antibodies. The absolute number of antibodies recognizing at least three or more genotypes was significantly higher in CLs as compared to CHRs (CLs:13 (IQR 2.2 - 53.0) versus CHRs: 0 (IQR 0 – 1.9), p = 0.03, Fig. 1B and Supplementary Fig. 2). Three out of eight CLs had antibodies that recognized all six HCV genotypes with a median frequency of 3.9 per 10,000 CD27+IgG+ B cells (IQR 3.4 - 14.6). Such broadly reactive antibodies were not detected in CHRs.

Cross-genotype antibodies from CLs predominantly target AR3.

To further characterize cross-genotype antibodies from CLs, we selected 113 B cell cultures from CL1, CL2, CL3, and CL7, who had the highest frequency of cross-genotype antibodies (Supplementary Fig. 3). To determine the epitopes targeted by these antibodies, we tested the antibodies secreted in the B cell supernatant by ELISA for binding to E2 alanine mutants in the four epitopes known to be recognized by broadly neutralizing HCV antibodies. Twelve alanine mutants were generated with mutations in epitope I (L413, G418 and W420), in AR3 / epitope II (L441, F442 and Y443), in AR3 / domain B (W529, G530 and D535) and in AR4 (R657, L692 and D698) [16,32,33]. Epitope specificity was determined by measuring the reduction in antibody binding to one or more

alanine mutants from the panel. An epitope was considered to be specifically recognized if an alanine mutation in that epitope resulted in more than 50% reduction of antibody binding compared to binding to wild-type E2.

In all four subjects, the majority of the cross-genotype antibodies (82/113, 73%) were specific for AR3 (Fig. 2A) as binding to the AR3-specific mutants (F442A, G530A and D535A) was reduced by more than 50% (Fig. 2B). Interestingly, binding of a subset of AR3-specific antibodies was not strongly affected by the alanine mutation in residue 441, which is in contrast to recent data from Gopal et al. who showed that this L441A mutation affected all AR3 antibodies [33]. Furthermore, we observed that three antibodies (C03, E17 and M11) found in subject CL2 were specific for an epitope shared by epitope I and AR3, as was evident by the loss of binding to the mutant W420A. This finding was confirmed at the monoclonal B cell level (Supplementary Fig. 4).

Besides antibodies specific to AR3, we observed that cross-reactive antibodies targeting AR4 were present in three CLs, as indicated by the decreased binding to the mutants R657A, L692A and D698A. Interestingly, these AR4-specific antibodies showed no or low binding to the soluble E2 ectodomain (Supplementary Fig. 5). Finally, in none of the CLs, we found antibodies that recognized epitope I or epitope II only.

Monoclonal cross-genotype antibodies from CLs are broadly neutralizing.

Thus far, antibody characteristics were determined using supernatants of B cells cultured at a density of 35 cells per well and therefore the supernatants of those

cultures could contain 34 irrelevant antibodies. Therefore, we analysed antibodies at the monoclonal B cell level to study the neutralizing potency of the antibodies in more detail. Focusing on cross-genotype antibodies against AR3 and AR4, thirteen mini-cultures with different alanine mutant patterns were selected, of which B cell clones were generated by single cell sorting (Supplementary material and methods). Immunoglobulin genes of AR3 and AR4 binding B-cell clones were sequenced and ten unique antibody sequences were identified (Fig. 3A). In addition, two additional monoclonal antibodies (AT1706 and AT1618) were isolated, from subject CL7 who had the highest frequency of cross-genotype antibodies when screening directly 5,000 CD27+IgG+ single cell cultures for HCVpp neutralization activity. All twelve antibodies were of the IgG1 isotype. In addition, the majority of AR3-specific antibodies (AT antibodies 1637-1640,1642,1643,1645) used a VH gene of the VH1.69 family. These results are in line with findings by us and others [15,26,34].

Next, we confirmed the cross-reactivity of the monoclonal antibodies by ELISA using the panel of eight E1E2 sequences from genotypes 1 to 6 (Fig. 3B). As expected from the mini-culture data, AT1644 and AT1645 were specific for only sequences from three different HCV genotypes only, whereas all other antibodies bound to E1E2 from genotypes 1 to 6.

The neutralization capacity of the antibodies was tested using the HCVpp system expressing E1E2 from genotypes 1a, 1b, 3a and 4d. These HCVpp were all relatively sensitive to neutralization by AR3B and AR4A (Fig. 4A). Except AT1644, which did not neutralize, all monoclonal antibodies neutralized at least

one HCVpp with ten antibodies showing broad cross-neutralization (Fig. 4B). All AR3-specific antibodies (AT antibodies 1637-1640,1642, 1643,1645 and 1706 - Supplementary Fig. 6) neutralized HCVpp with an 50% inhibitory concentration (IC_{50}) range from 0.088 to 1.41 µg/mL (Fig. 4B), while the AR4-specific antibodies (AT1644, AT1646, AT1647 and AT1618 - Supplementary Fig. 6) had IC_{50}'s above 0.1µg/mL (Fig. 4B).

The neutralization capacity of the antibodies was further tested using a panel of four HCVpp which have been shown to be resistant to antibody-mediated neutralization (Fig. 4C) [35]. Eight out of twelve antibodies (AT antibodies 1637-1640,1642, 1643,1618 and 1706) neutralized at least one resistant HCVpp with an IC_{50} range of 0.39 to 18.2 µg/mL (Fig. 4D). Of these antibodies, AT1640 and AT1618 neutralized all eight HCV isolates (Fig. 4B and Fig. 4D). Importantly, by examining the combined neutralization profiles of the antibodies per subject, antibodies from three of the four subjects tested (CL1, CL2 and CL7) covered all tested HCVpp.

To confirm the neutralization capacity of the antibodies, AT antibodies were tested at 20µg/ml for neutralization of HCVcc (isolate H77). Apart from AT1645, all AR3-specific antibodies (AT antibodies 1638-1640, 1643 and 1706) neutralized infection by more than 50% (Fig. 5A). In addition to AR3 specific antibodies, neutralization activity above 50% was observed for the AR4-specific antibody AT1618. These finding were consistent with the data obtained using HCVpp.

Since AT1618 targets AR4, and we showed that it is able to neutralize all HCVpp from our panel, including the resistant ones, we wanted to confirm its breadth in the HCVcc system using the JFH1 prototype (genotype 2) and ED43 HCV chimera (genotype 4a). We found that AT1618 neutralized both viruses with IC_{50} 's of 0.27 µg/ml and 0,080 µg/ml, respectively (Fig. 5B and 5C).

As the expression of HCV entry factors most probably differs between Huh-7 cells and primary human hepatocytes, we repeated HCVpp neutralization experiments using primary hepatocytes. At least one antibody from each subject was selected for this experiment (AT1638, 1637, 1642, 1706 and 1618). As shown in Fig. 6A, at a concentration of 0.5µg/ml, all antibodies neutralized genotype 1a H77 HCVpp infection by more than 50% using primary hepatocytes. Moreover, AT1638, 1637 and 1642 also reduced the infection of AMS 3a.26 HCVpp by more than 50% (Fig. 6B). Infection of AMS 3a.26 HCVpp was reduced by AT1706 and AT1618, however, by 25% to 50%. This reduction was consistent with data from the neutralization experiments using Huh-7 cells, where the IC_{50} for AMS3a.26 HCVpp was respectively >1.36 and 1.64 µg/ml (Fig. 4B).

DISCUSSION

For the design of a protective HCV vaccine, a detailed understanding of the mechanism by which HCV infection is spontaneously cleared, is crucial. A strong and broad T cell response has been associated with clearance of HCV (re)infection [6,7]. However, the role of B cells producing HCV neutralizing antibody is largely undefined. Here, we studied the B-cell repertoire against HCV

E1E2 in a cohort of persistent IDU following clearance of primary HCV infection. We compared the breadth of the antibodies against E1E2 early after infection between subjects who were HCV RNA negative at the end of study follow-up (CLs) and subjects who ultimately became chronically infected (CHRs). Our main finding is that the frequency of B cells producing cross-genotype antibodies was significantly higher in CLs compared to CHRs. Moreover, in a subset of four CLs who we studied in more detail, we found that these cross-genotype antibodies mainly recognized AR3 and showed broad neutralizing activity. In addition, in three of these four CLs, we also identified cross-genotype antibodies targeting AR4.

The role of cross-neutralizing antibodies in protection against HCV infection in real-life settings has rarely been studied. Pestka et al. were the first to demonstrate that polyclonal serum neutralizing antibody responses were associated with spontaneous clearance of HCV infection [8]. Recently, it was shown, that cross-neutralization of a genotype 1 HCVpp panel by serum from subjects infected with genotype 1 and non-1 viruses, was associated with HCV clearance [9]. From two of these subjects, broadly neutralizing antibodies were isolated [34]. In the study presented here, a high frequency of B cells producing cross-genotype antibodies early after infection was associated with clearance of HCV infection. Moreover, in four of these subjects with such strong cross-genotype B-cell response, we isolated broadly neutralizing antibodies. It is

tempting to hypothesize that these B cells expressing broadly neutralizing antibodies were induced during infection and contributed to viral clearance.

It is important to note, however, that we do find variation in both breadth and frequency in the antibody responses between CL subjects. Subject CL5 for example presented only with genotype-specific antibodies, which could be due to the time point of PBMC selection or indicate that other components of the immune system such as T cells may have played a role in viral clearance. In addition to difference in breadth, we observed a relatively large difference in the frequency of E1E2 specific antibodies (IQR: 3.6 – 77 antibodies per 10,000 antibodies). This could be a consequence of the time point of PBMC selection, natural inter-donor variation or technical issues.

The longitudinal design of our study allowed the selection of a subgroup of cohort participants (CLs) who never became chronically infected, despite presumably frequent re-exposure, since they continued injecting drugs during a long period in which incidence of infection was very high in IDU [20]. Indeed, in three of these CLs reinfections were documented after the time point of PBMC collection, even though HCV RNA testing, with a median of 11 HCV RNA tests per case [22], was not performed regularly enough to diagnose all reinfections [36]. Despite this assumed frequent re-exposure, CLs were HCV RNA negative at the end of a median follow-up of 17.7 years and developed memory B cells expressing broadly neutralizing antibodies. This finding is consistent with previous reports by

us and others describing memory B cells expressing broadly neutralizing antibodies from IDU who did not become chronically infected for more than 5 years following clearance of primary infection [26,34]. Importantly, we also showed that such B-cell response can be long-lasting, since memory B cells expressing broadly neutralizing antibodies were circulating 25 years after clearance of the primary infection [26]. In this study, we confirm the longevity of memory AR3-specific B cells as these B-cells were still circulating 5.2 years after clearance of the primary infection in the CL3 subject (Supplementary Fig. 7). Taken together, this suggests that broadly neutralizing antibodies induced during primary infection, also contribute to the prevention of the subsequent chronic infections. However, such broadly neutralizing antibodies may not always provide sterilizing immunity, as indicated by the reinfections we observed in CLs.

The majority of antibodies with cross-reactivity against at least three genotypes, recognized AR3. From the four CLs with the largest number of HCV specific B cell cultures, we isolated eight AR3-specific monoclonal antibodies and confirmed their broadly neutralizing properties. This finding is consistent with other studies where AR3-specific antibodies have been isolated from subjects who cleared all infections [26,34]. The dominance of AR3-specific antibodies in the B-cell repertoire from CLs suggests that these antibodies play an important role in protection against HCV infection.

Besides AR3-specific antibodies, we also detected cross-genotype antibodies targeting AR4. Little is known about AR4, since the focus has mainly been on the antibody AR4A [16]. While it has been suggested that AR4-specific antibodies are rare, we observed that 27 out of 113 (24%) cross-genotype antibodies targeted AR4, and that these antibodies were present in three of the four CLs. Although less abundant than AR3 antibodies, this suggests that AR4 targeting antibodies may be present in most subjects who develop cross-reactive antibodies. However, in contrast to the broad neutralization of AR4A, only one out of the four broadly reactive monoclonal AR4-specific antibodies isolated by us, showed broadly neutralizing capacity (AT1618). Consistent with previous observations for AR4A [16], we found that AR4-specific antibodies did not bind to soluble E2 ectodomain suggesting that AR4-specific antibodies only bind to the E1E2 complex or, alternatively, that differential conformation of soluble E2 precludes the binding of AR4A-like antibodies.

Several studies have shown that AR3- and AR4-specific antibodies act synergistically [16,37] and importantly, antibodies targeting AR3 (AR3A, AR3B) protected mice against HCV infection in combination with antibody AR4A [17]. In this light, our observation that three out of four CL subjects developed both AR3- and AR4-specific antibodies is interesting, since it may suggest a similarly combined neutralizing role of AR3 and AR4 specific antibodies in their clearance of HCV. If this is confirmed in future studies, a vaccine should induce antibodies against these two antigenic regions.

Although we did not detect antibodies recognizing all genotypes in CHRs, others did isolate broadly reactive antibodies targeting AR3 from chronically infected subjects [15,38]. However, these antibodies were only found relatively late after infection. Here, we found that CLs had already developed B cells expressing cross-reactive antibodies within one year after acquiring HCV, while the CHRs at the same time point had mainly developed genotype-specific antibodies. This is in line with Osburn *et al.* who showed that the development of cross-reactive antibodies is delayed in chronically infected subjects [9]. Interestingly, one CHR (CHR2) had four cross-reactive antibodies which were specific for only 2 to 4 genotypes (Figure 1B). We found that those cross-reactive antibodies targeted AR3 (Supplementary Fig. 8).Taken together, this suggests that in chronically infected individuals, AR3-specific antibody response may be too weak and/or may develop too late to prevent chronic infection. If confirmed, this means that a strong and broadly neutralizing antibody response should be established very early after infection in order to confer protection.

For the early development of cross-reactive antibodies, the host genetics may also be crucial since it was shown that early appearance of cross-reactive neutralizing antibodies was associated with two SNPs located upstream of the HLA-DQB1 and HLA-DQA2 genes [9].

In conclusion and to our best knowledge, this study describes for the first time, the B-cell repertoires against HCV E1E2 from subjects who spontaneously

cleared one or multiple infection(s) in comparison to subjects who ultimately developed a chronic infection. Our data suggest that an early and strong B-cell response inducing broadly neutralizing antibodies against AR3 and AR4, is important for viral clearance and prevention of subsequent chronic infections upon re-exposure. These observations permit cautious optimism that development of an effective preventive vaccine is feasible.

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Table

Table 1. Demographics and clinical characteristics

Subject ID	Type of HCV infection(s)	Age*, (yr)	Gender	Follow-up #, (yr)	Duration of injecting drug use#, (yr)	HCV sero-conversion	Calendar year of infection	Timepoint of PBMC collection, (yr)
CHR1	Chronic upon primary	34	M	12	4.4	Sc	1989	1.12 #
CHR2	Chronic upon primary	21	F	11	0.2	Sc	1988	1.18 #
CHR3	Chronic upon primary	47	M	17	11.9	Sc	1993	1.05 #
CHR4	Chronic upon reinfection	24	M	21	13.2	Rc	1989	0.81 #
CHR5	Chronic upon reinfection	29	F	18	4.9	Rc	1986	4.33# 1.19 $

CL1	No reinfection Cleared	29	F	18	6.6	Rc	1992	0.47 #
CL2	multiple infections Cleared	23	F	17	7.5	Rc	1989	0.13 #
CL3	multiple infections Cleared	39	M	19	2.1	Sc	1990	0.43 #
CL4	multiple infections No	20	F	19	5.3	Rc	1989	0.59 #
CL5	reinfection Cleared	29	F	9	3.2	Rc	1988	0.75 #
CL7	multiple infections Cleared	23	M	23	14.5	Rc	1986	9.36#, 2.51 $
CL8	multiple infections Cleared	33	F	14	6.4	Rc	1989	0.93#, 0.59 $
CL9	multiple infections	26	M	7	4.9	Rc	1987	5.28#, 0.54 $
Median		29		17.5	5.3		1989	0.75

CHR: Subjects who ultimately became chronically infected; CL: Subjects who, at the end of study follow-up, were HCV RNA negative; Chronic upon primary: subjects who became chronically infected following primary infection; Chronic following reinfection: subjects who became chronically infected following reinfection; Cleared multiple infections: subjects who spontaneously cleared both primary infection and reinfection; No reinfection: subjects who spontaneously cleared primary infection without a documented reinfection; yr, years; F, female; M, male; Sc, seroconverter; Rc, recent; yr,years; *, at the estimated date of infection, #: after the estimated date of infection and $, after estimated time of reinfection

Figure legends

Fig. 1. Frequency and cross-reactivity of E1E2 specific antibodies.

The number and the cross-reactivity of E1E2 specific antibodies were studied in subjects ultimately chronically infected (CHR) or in subjects who, at the end of study follow-up, were HCV RNA negative (CL). Antibodies present in B cell supernatant of 35 cell per well mini-cultures (total of 10,290 CD27+IgG+ B cells) were tested for binding to E1E2 derived from 6 different genotypes using a multiplexed flow cytometry assay. (A) From each subject (in row), the number and frequency of antibodies specific for E1E2 from genotype 1 to 6 / isolate (in column) are indicated. The grey filling colours indicate the number of E1E2 antibodies targeting E1E2 of a particular genotype: white indicates the lowest number of antibodies and grey dark the highest. For each subject, the highest number of antibodies binding E1E2 from a particular genotype was bolded and underlined. NA: Not Applicable; ND: Not Determined. (B) The pie chart with grey scale represents cross-reactivity of the E1E2 specific antibodies from each subject.

Fig. 2. Epitope binding of the cross-genotype antibodies from CL subjects.

To determine the epitopes targeted by the cross-genotype antibodies, antibody binding was tested by ELISA using E1E2 mutants. Single alanine amino acid substitutions were introduced in the E1E2 sequence from isolate H77 at positions important for binding of broadly neutralizing antibodies in four described epitopes (epitope I, AR3 / epitope II, domain B and AR4). Cell lysates from 293T cells

transfected with the alanine-mutants of E1E2 were incubated on *Galanthus nivalis* lectin pre-coated wells before the antibodies containing B cell supernatants from subjects CL1, CL2, CL3 and CL7 were added. From the optical density value of each well the background was subtracted, and the relative binding was calculated by dividing the value obtained on wild-type protein by the alanine-mutant protein value. (A) The pie charts represent the distribution of epitopes targeted by all cross-genotype specific antibodies from each subject separately. (B) The colour filling indicates the relative binding level: black 0% - 25%, grey 25-50% and white 50% -150%. The E2 mutants were designated X123Y where 123 is the residue position, X is the wild-type amino acid residue in H77 and Y indicates the replacing amino acid. The data are the mean values of two experiments performed in duplicate.

Fig. 3. Characteristics of monoclonal cross-genotype antibodies from CL subjects.

(A) Shown are antibody heavy and light chain genes of ten B cell clones that were isolated by single cell sorting of B cells from subjects CL1, CL2, CL3 and CL7, and two B cell clones (AT1706 and AT1618) from subject CL7 that were obtained after screening single B cell cultures for HCVpp neutralization activity. Gene analysis was done using the IMGT/V-QUEST database. VH: variable domain of the heavy chain, VL: variable domain of the light chain, CDR: complementarity determining region, aa: amino acids. (B) The cross-reactivity of the twelve monoclonal antibodies was confirmed by ELISA. First, the cell lysates

of 293T cells transfected with E1E2 from genotype 1 to 6 or non-transfected cells were captured on GNA pre-coated wells before antibodies present in B cell supernatants were added and detected using anti-human IgG conjugated horseradish peroxidase. The x-axis indicates the mean optical density (OD) at 450nm and the errors bars represent one standard deviation. Anti-RSV F antibody D25 was used as a negative control and anti-HCV AT12-009 as a positive control. The assay was performed in duplicate wells and repeated at least once.

Fig. 4. Neutralization activity of cross-genotype antibodies from CL subjects in HCV pseudotyped particles (pp) system.

The neutralization capacity of the antibodies was tested using (A-B) a panel of four HCVpp from genotypes 1 to 4 and (C-D) a panel of four HCVpp resistant to antibody neutralization genotype 2 to 6. Neutralization was determined by incubating HCVpp expressing E1E2 with a two-fold serial dilution of antibody-containing B cell supernatants before inoculation of Huh-7 cells. The 50% inhibitory concentration (IC_{50} in µg/mL) was determined as the midpoint between the last concentration giving more than 50% neutralization and the first concentration giving less than 50% neutralization. The grey colours indicate the range of neutralization: an IC_{50} <0.1 µg/mL (black), 0.1–1 µg/mL (dark grey), 1–10 µg/mL (light grey) and above 10µg/ml or no 50% neutralization was observed (white). ND: not done. The genotype of the isolates is indicated between brackets.

Fig. 5. Neutralization activity of cross-genotype antibodies from CL subjects in HCV cell culture (cc) system.

(A) Neutralization was determined by incubating HCVcc expressing E1E2 from isolate H77 with 20μg/mL of purified antibodies before inoculation of Huh-7.5 cells. Percentage of neutralization was determined by comparison to the number of cells infected in the absence of antibodies. Anti-tetanus toxin antibody was used as a negative control and anti-HCV AR3B as a positive control. (B) Neutralization potency of AT1618 was determined by incubating HCVcc expressing E1E2 isolate JFH-1 (genotype 2a) and ED43 (genotype 4a) with a four-fold serial dilutions of purified antibody starting 50μg/ml before inoculation of Huh-7.5 cells. The neutralization percentage was determined by the reduction in luciferase activity compared to background and no antibody samples. 50% inhibitory concentrations (IC_{50} in μg/mL) were determined using non-linear regression analysis. Anti-HIV antibody 3BNC60 was used as a negative control and anti-HCV AR4A as a positive control. ND: not done. The errors bars represent one standard deviation (SD).

Fig. 6. AT antibodies to block entry of HCV into primary hepatocytes.

The neutralization capacity of the antibodies was tested using HCVpp derived from (A) H77 HCV isolate (genotype 1), or (B) AMS3a.26 HCV isolate (genotype 3). HCVpp supernatants were incubated with 0.5 μg/mL antibody-containing B cell supernatants before being inoculated onto primary human hepatocytes

seeded the day before. After three days, the luciferase activity was measured using an EnVision® Multilabel Reader. The anti-RSV F antibody (D25) was used as a negative control. The y-axis indicates the mean relative light units and the errors bars represent one SD. The grey dotted line depicts 50% of infection based on the negative control.

Highlights

- HCV clearance is associated with high number of cross-genotype specific antibodies.
- Antigenic region 3 (AR3) is the dominant epitope recognized by the antibodies.
- AR3-specific antibodies neutralized HCV pseudoparticles and cell culture viruses.
- A large number of clearers develop antibodies targeting antigenic region 4 (AR4).
- AR4-specific antibody AT1618 also neutralized neutralization-resistant variants.