CHRONIC HEPATITIS C VIRUS INFECTION IN CHIMPANZEES
-prevention & consequences-

Babs Verstrepen

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-preventie & consequenties-

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Chronic Hepatitis C Virus Infection in Chimpanzees, Prevention and Consequences

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PREFACE

With the near disappearance of chimpanzees as an animal model on one side, and the approval of direct acting antiviral drugs by regulatory agencies on the other, this thesis is positioned in an era of changing perspectives in HCV research. It summarizes the key-role of chimpanzees in HCV research so far. This thesis elaborates on potential mechanisms behind successful prophylactic vaccine candidates, but also the consequences of chronic HCV are discussed.

GENERAL INTRODUCTION

Chronic hepatitis, caused by persistent infection with hepatitis C virus (HCV) is a major health concern worldwide [1]. The number of chronic HCV carriers is estimated to be 170 million, about 1 to 2% of the total world population **(Figure 1)**. The virus was first described in 1989 [2]. Before reliable diagnostic tests were introduced, HCV was primarily spread via blood transfusions [3].

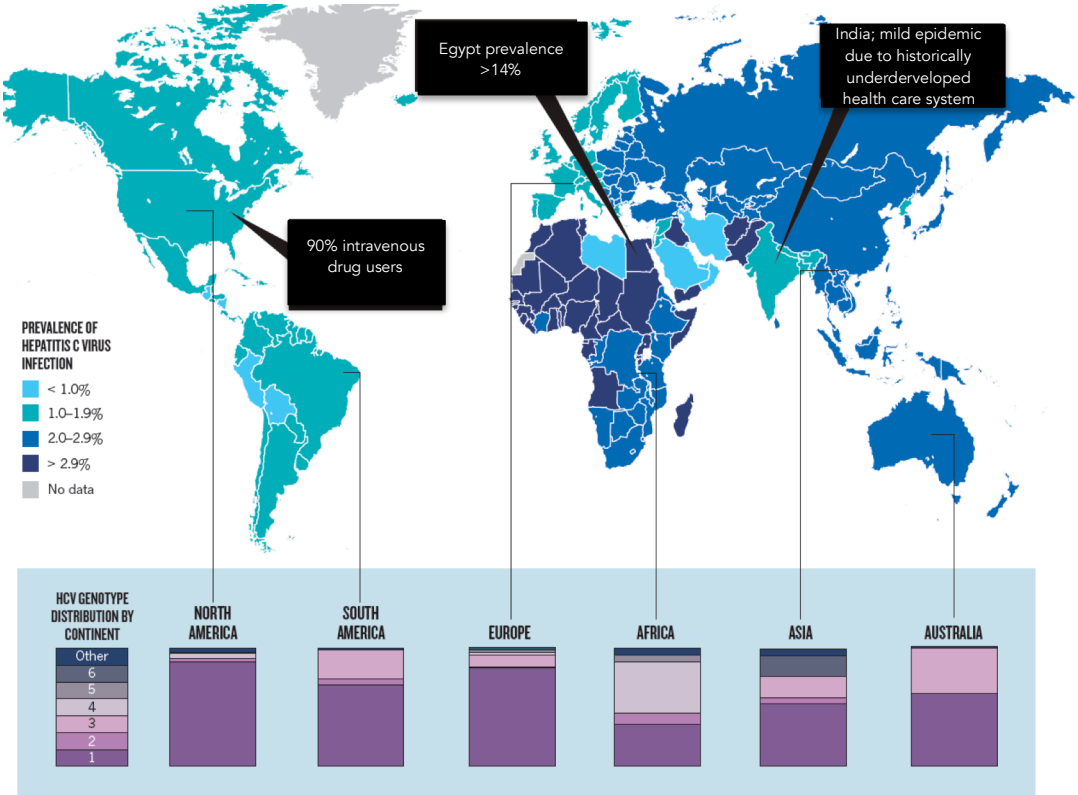

Figure 1; World wide prevalence of HCV and subtypes
Adapted from Gravits et al [1]

Life cycle of HCV

HCV is a 9600 nucleotide, single-stranded positive-sense RNA virus that belongs to the Flaviviridae. The structural proteins form the virus particle, while the nonstructural proteins are involved in replication, maturation and assembly [4] **(Figure 2)**.

The surface of an infectious HCV particle is composed of a lipid bilayer with the envelope glycoproteins. Inside are the viral Core protein and viral RNA. After cell entry, host machinery is utilized to translate viral RNA into one large precursor polyprotein. The structural proteins Core, E1 and E2 are separated from the nonstructural proteins NS1, NS2, NS3, NS5A and NS5B by p7, a presumed viroporin [5]. During and after the translation process, host and viral-encoded proteases cleave the polyprotein into 10 mature viral proteins. Post-translational processing is orchestrating the heterodimeric complexing of NS3 and NS4A proteins (NS3/NS4A) where NS3 has proteolytic activity and NS4 acts as a cofactor [6].

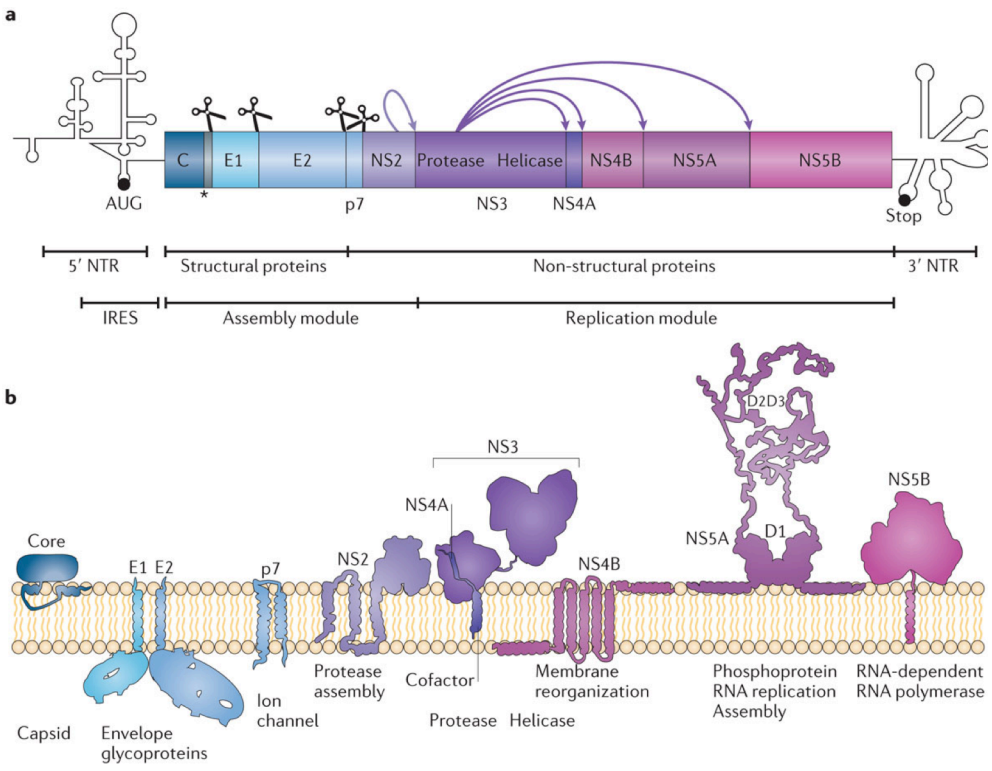

Figure 2; HCV genome and translation.
Adapted from Bartenschlager et al [2]

Genetic diversity HCV

There are seven major genotypes of HCV [7, 8]. Each genotype consists of a cluster of different subtypes, and within each patient, closely related quasi-species are present. The difference between two distantly related HCV isolates can be as high as 30% at the nucleotide level [9]. The genetic variation of HCV has implications for the development of a successful vaccine. In addition, the circulating quasi-species can easily adapt, especially in the hypervariable regions (HVR) in the envelope proteins, and are notorious for escaping the immune system as well as drugs that are used for treatment.

Transmission

Transmission of HCV occurs via blood-blood contact. Nowadays in the western world, the majority of the new infections are associated with intravenous drug use. Not only via the sharing of contaminated needles but also other injection-equipment [10]. Still, there are several examples of drastically declining numbers of new HCV cases, after the introduction of surveillance programs and the distribution of fresh disposable needles amongst intravenous drug users [11, 12].

In other geographical regions, the mode of transmission is different. The situation is especially worrying in Egypt, where an estimated 12% of the population is infected with HCV. The high prevalence is the result of an unsafe treatment-procedure of an endemic schistosomiasis infection during the years 60-80s of the last century. Currently, the infrastructural organization of the Egyptian health care system is, at least partially, responsible for ongoing transmission in the region [13]. Recently, WHO has declared the large reservoir of chronic HCV carriers a serious risk, as tourism and migration can contribute to spreading of the virus to places outside the region.

Chronic infection

HCV infection is characterized by a high propensity for the development of life-long viral persistence. Only 25% of the acute infections is spontaneously eradicated, normally within the first six months after infection [14]. During acute HCV infection, clinical symptoms are mild or even absent. For that reason acute HCV infections are often unrecognized. When acute HCV infection develops into a persistent infection, the majority of the patients develop chronic hepatitis and over decades the virus causes subtle progressive hepatic damage. Ultimately, this may lead to cirrhosis, decompensating liver

congestion or hepatocellular carcinoma **(Figure 3)**. It has been calculated that, worldwide, 27% of the cases of liver cirrhosis can be accounted for by HCV and overall, persistent HCV infection accounts for 3 million deaths each year (source WHO).

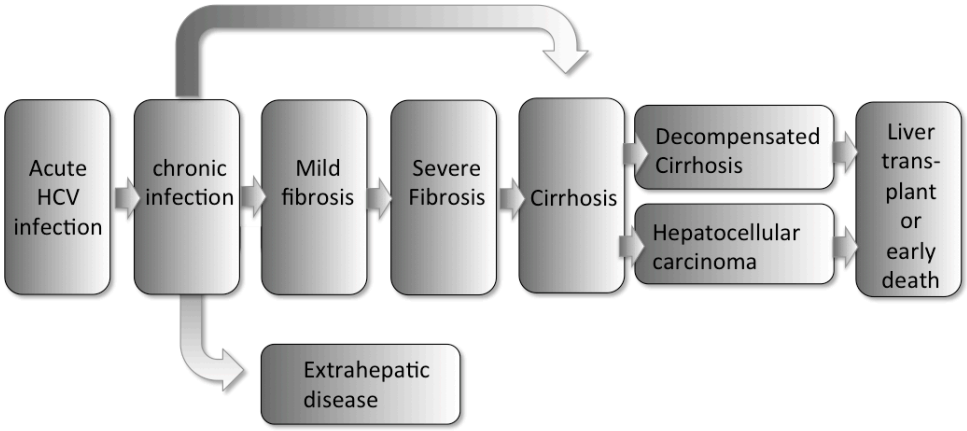

Figure 3; Schematic overview course HCV infection.
The course of HCV infection is very heterogeneous. Depending on age of, genotype, lifestyle and many other factors, HCV infection can run an aggressive course or a more mild course, with severe or mild pathophysiological damage in the liver. The time between infection and actual clinical symptoms is sometimes more than 30 years.

Therapeutic drugs or a vaccine?

Soon after the discovery of HCV, it was clear that investigating this virus was going to be an even bigger challenge compared to HIV. Many of the tools that were available for HIV research, were not available for HCV. HCV was found to have a narrow host range; only humans and chimpanzees were susceptible for infection. Unlike the S(H)IV model for HIV infection in humans, there was no surrogate model available for HCV. And, before 2003, there was no *in vitro* method available to culture HCV. The lack of these tools, and the unknown virus' life cycle, made it clear it would be sheer impossible to develop specific therapeutic antiviral drugs. For that reason, it was believed that the development of a vaccine would be a more efficient approach to fight the HCV-epidemic.

Chimpanzees in biomedical research

Humans and chimpanzees (*pan troglodytes*) share a common ancestor who lived approximately 30 million years ago, before the hominoid lineage split. Chimpanzees are humans' closest living relatives with 98.9% identity at DNA level [15]. Since the 40s of the last century, chimpanzees have been used in research. First in the US space program and later also in biomedical research. The colonies of chimpanzees in research facilities were founded from animals that were imported from the wild in Western Africa. Soon, breeding programs assured enough offspring for experimental work. Facilities became self-sustainable and no longer required import of chimpanzees from the wild.

Public concerns about research with non-human primates, in particular chimpanzees, has eventually led to the total ban of the use of apes for research in Europe, and a significant reduction of the number of animals used in the US [16].

Chimpanzees and HCV research

No doubt, chimpanzees have been the most important animal model to study HCV [17]. In the late 80s, after it became clear that the majority of blood borne chronic liver inflammations was not caused by hepatitis A or B virus, serum from a non-A-non-B hepatitis patient was inoculated into a chimpanzee [2]. From this chimpanzee, a cDNA bank was derived and in 1989 Michael Houghton and his coworkers at Chiron Inc., identified HCV as the main causative agent for non-A-non-B hepatitis [2].

To date, the chimpanzee is the only validated animal model to study immunity associated with acute resolving infection, and protective immunity against HCV reinfection. Over the past 35 years, experimental HCV infections of chimpanzees has provided groundbreaking information regarding the identification, characterization, transmission and early responses after HCV infection. Studies in chimpanzees have enabled us to identify immune mechanisms that are associated with viral clearance or chronic infection. This was critical for the design of prophylactic vaccine candidates. Subsequently, chimpanzees were used to evaluate the efficacy of vaccines and different vaccination strategies.

Primary HCV infection in chimpanzees

To be able to study the effect of a vaccine or vaccination strategy, it was necessary to identify the virological characteristics of HCV without any intervention. There are numerous reasons why it is difficult to study early

events in HCV infection in humans. Firstly, the vast majority of the acute HCV infections are asymptomatic and patients therefore rarely seek medical attention. Secondly, collecting serial blood samples and occasional liver-biopsy material from one individual during acute HCV infection is very difficult. Additionally, collecting pre-exposed bio-specimen from the same patient was/is nearly impossible, especially since the high-risk groups for contracting HCV had not been identified at that time. Therefore, experimental inoculation of chimpanzees was pivotal to be able to study early virological and immunological events.

In chimpanzees, similar to humans, intravenous exposure to HCV can lead to either a transient self-limiting infection or it may develop into a persistent infection [18]. In both humans and chimpanzees, viral RNA is detectable by RT-PCR in plasma and liver tissue [19]. In addition, anti-HCV antibodies appear in peripheral blood of both species 6 to 8 weeks after HCV exposure [20, 21]. In the majority of human individuals, antibodies remain detectable in blood after viral clearance, while in chimpanzees sometimes a gradual loss of HCV specific antibodies after viral elimination has been reported [19, 22]. However, in humans, HCV specific cellular immune responses have been observed in seronegative individuals, implying that in humans the loss of HCV-specific antibodies after viral clearance also occurs [18, 23-25].

Published data on cellular immune responses showed that HCV specific CD4 and CD8 T-cell responses in both humans and chimpanzees are weak after infection. Spontaneous clearance was associated with relatively strong cellular responses compared to the individuals that became persistently infected [26-30]. Also in liver biopsies from HCV infected patients and chimpanzees, virus-specific CD4 and CD8 T-cells were observed [31-34]. Relatively strong liver-associated T-cell responses were associated with viral clearance [34].

HCV reinfection in chimpanzees

Documented reinfection studies in humans are relatively sparse [35-38]. Longitudinal analysis of human intravenous drug users were performed, but results were inconclusive as to whether a previously cleared HCV infection induces functional immunological memory [14, 36, 37]. Important insights were obtained from chimpanzees in which experimental HCV re-exposure was studied in a controlled setting as far as genotype, dose and route of infection, and longitudinal follow up studies could be performed [39-46].

Reinfection studies in chimpanzees have demonstrated that all of the three possible outcomes: i.e. protection from infection [43, 44], protection from viral persistence [39, 43-45] and persistent HCV infection [39], can occur. Pairwise comparison of virological parameters during primary infection versus chimpanzees that were rechallenged, showed that previous HCV clearance provided some protection [47]. This was characterized by reduced duration, peak virus load and reduced frequency of development of persistent HCV infection [47].

Understanding the underlying mechanisms that contribute to protection against chronic infection has been an important research goal, and was pivotal for further HCV vaccine development. Since HCV-induced liver damage only leads to a fatal condition after decades of ongoing immunopathogenesis, a vaccine achieving a similar rate of protection from chronic infection as observed after reinfection, would already be of great value.

Viral persistence in humans and chimpanzees

The vertebrate immune system comprises two interrelating systems, the innate and the adaptive immune system. The innate system is rather rigid and partly orchestrated by the genome. Adaptive immunity, with its variability and memory, is based on somatic gene rearrangements, and the repertoire of antigen-specific receptors is presented on the surface of immune cells. The intense communication between the innate and adaptive immune system is regulated via soluble chemokines and cytokines, and the expression of cell-associated receptors. Taken together, this enables the recruitment of specific effector cells to the site of infection. In order for a pathogen to survive and persist in this hostile environment, it needs to evade the immune system of the host.

Based on antibody data, the WHO estimates that 55 to 85% of the HCV infections eventually develop into a persistent infection. The documented percentages of chimpanzees with persisting HCV infection varies between different laboratories from 39 to 70% [22, 47-49]. This indicates that HCV is very successful in evading immunity. In the following paragraphs, the main evasion strategies of HCV will be discussed.

Immune correlates

A schematic overview of the immune mechanisms during HCV is shown in **Figure 4**.

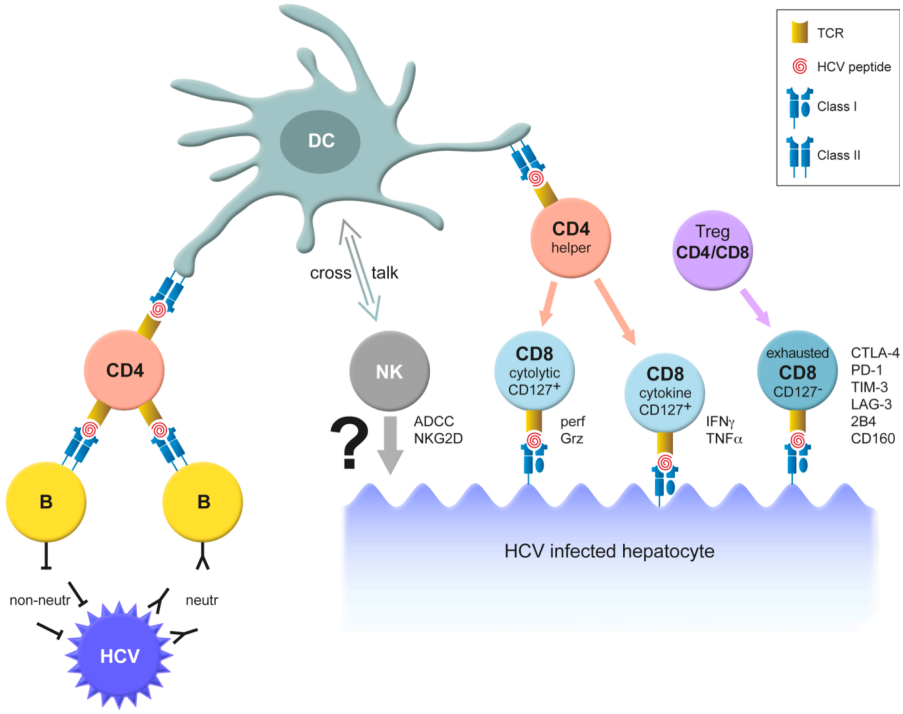

Figure 4; Schematic overview of cellular effector responses during HCV infection. Antigen presenting cells (DCs) or Kupffer cells in the liver, present HCV peptides in the context of MHC class II molecules to the T-cell receptor (TCR) on CD4 T-cells. CD4 cells may activate B-cells. Antibodies produced may be neutralizing, bind to circulating HCV particles and prevent the infection of new hepatocytes. Or the antibodies may be non-neutralizing and potentially play a role in ADCC. CD4-helper T-cells can also stimulate cytolytic T-cells (CTL). CD8 T-cells may be directly responsible for lysis when they produce degranulation molecules like granzymes or perforin after the recognition of a peptide on the surface of an HCV infected hepatocyte. Or indirectly, mediated by secretion of cytokines. CD8 T-cells affected by Tregs or express markers that are associated with exhaustion. In that case CD8 T-cells are functionally impaired and incapable of lysing HCV infected hepatocytes.

A) Immunogenetics.

MHC class I genes

The main function of MHC class I and class II molecules is the binding of pathogen-derived peptides, and present them to CD8 and CD4 effector T-cells. In humans, the outcome of HCV infection is associated with protective HLA class I alleles HLA-B27, HLA-B57 and HLA-A2, and class II molecules HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*1101 and HLA-DRB1*0301 [50-53].

Although the exact same MHC class I alleles that are associated with HCV clearance are not present in chimpanzees, homologues with similar peptide-binding characteristics have been identified in these animals [54]. All these findings may be important in the development of a successful vaccine.

Interferon stimulating genes

Genome wide association studies in humans have shown that spontaneous clearance, and better chance of sustained virological response after IFNα treatment, is associated with polymorphism near the IFNλ, IL-28B gene [55, 56]. Although, *in vitro* studies have shown that IFNλ is produced by hepatocytes [57, 58] in the liver. different genotypes do not have a direct regulatory effect on the transcription of IFNλ, as shown by similar levels of the protein in serum [59]. They do however correlate with serum levels of another IFNγ induced protein, IP-10 [60, 61]. If or how these pathways interrelate, and how innate effector functions are affected, is still under investigation.

In chimpanzees, similar mechanisms may play a role. Not only have there been reports on up-regulation of intrahepatic ISGs in HCV infected chimpanzees [59, 62], it was also reported that ISG up-regulation was accompanied by robust intrahepatic responses of natural killer (NK) cells in a chimpanzee reinfected with HCV [63].

KIR genotypes

The lymphocyte population in the liver is enriched with NK cells, and NK cells play an important role in host defense against viral infections. In humans, the role of NK cells during HCV infection and HCV-associated liver damage is ambiguous (reviewed [64]). NK cells are associated with hepatic fibrosis, while on the other hand it was demonstrated that NK cells have anti-fibrotic properties, and that they control HCV replication by killing HCV infected hepatocytes. Additionally, successful antiviral therapy was found to be associated with activation of NK cells.

The activation of NK cells is partly regulated at genetic level. Killer cell immunoglobulin-like receptors (KIR) are members of the CD158 gene family. KIRs are part of the extensive repertoire of receptors expressed by NK cells [65]. Depending on the presence of immune tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain, NK receptors are either inhibitory or activating. KIRs bind to MHC class I molecules, and in this respect, MHC molecules can be divided into two groups. C1-KIRs contain an asparagine at position 80 in the α1 domain, while C2-KIRs contain a lysine at this position. The combinatorial

expression of class I molecules and KIRs determine the development and activation of NK cells.

In humans, homozygosity for C1 type HLA-C, in combination with the inhibitory KIR2DL3, is associated with HCV clearance [66]. In chimpanzees, also a relation between KIR and MHC class I was found. Homozygous C1 type Patr-B combined with Pt-KIR3DS2 or Pt-KIR2DL9 is associated with HCV persistence [67].

B) Innate immune cells

Dendritic cells

The main function of immature dendritic cells (DCs) is to scan the environment for antigens of potential pathogens. Internalization of the antigen leads to DC maturation and migration to the draining lymph node. Meanwhile, the antigen is processed and subsequently presented to T- and B-cells. DCs are innate immune cells that orchestrate and modulate adaptive immune responses.

Documentation of phenotype and function of DCs during chronic HCV infection in humans is contradictory. Several papers reported no changes [68-71] while others showed impaired function of DC during HCV infection [72-77]. It was hypothesized that this impairment is one of the key-determinants in facilitating HCV persistence. Analysis of DC function in chimpanzees showed that the function of monocyte derived DCs was maintained during acute and chronic infection [78, 79]. Therefore it can be concluded that impaired DC function is not the only mechanism leading to virus persistence.

In addition, the IFNα-producing capacity of one particular subset of DCs, plasmacytoid DC (pDC), was studied in human patients during acute and chronic HCV infection [80]. Here, it was found that both the frequency, as well as IFNα-producing capacity of pDCs were reduced during acute HCV infection and remained poor during chronic HCV infection. There is no data available on the frequency and function of pDCs in HCV infected chimpanzees.

NK cells

NK cells are innate immune effector cells with the ability to lyse infected target cells without prior activation. NK cells are also recognized for their immune modulatory function that influence downstream adaptive immune responses [81, 82]. Communication between NK cells and other immune cells is regulated via a wide range of soluble factors like chemokines, cytokines and surface associated receptors [83]. On the cell surface of NK cells both activating and inhibiting recognition-structures are presented. Not only the earlier

discussed KIRs, but also the so-called natural cytotoxicity receptors (NCRs), play an important role in NK activation [84]. NK cells are important immune cells in the first line of defense to several viral infections. Many have reported dys-balanced NCR expression and impaired NK function in relation to HCV infection but the results are inconclusive [85-92]. Although microarray analysis of an HCV infected chimpanzee demonstrated up-regulation of NK-associated genes [93], chimpanzee NK cells have been poorly studied so far [63]. This is mainly due to the ubiquitous expression of CD56 on chimpanzee NK cells that prevents the identification of $CD56^{bright}$ and $CD56^{dim}CD16^{pos}$ subsets in these animals.

C) Adaptive immune cells

As schematically depicted in **Figure 5**, four different scenarios can be used to describe the course of HCV infection and the specific adaptive immune responses. 1) Spontaneous clearance of HCV infection **(Figure 5A)**; associated with early and effective T-cell responses. This successful immune response is characterized by relatively strong expansion of T-cells. These T-cells are fully functional with respect to cytolytic capacity as reflected by granzyme, perforin secretion and/or cytokine production [94-102]. 2) Transient immune control **(Figure 5B)**; characterized by ensuing viral escape, possibly as a result of either immune mediated viral selection or tolerance/immune exhaustion. Immune pressure may drive the generation of virus variants in which relevant T- or B-cell epitopes are mutated. Epitopes are therefore no longer recognized by immune effector cells when they are presented on the surface of infected hepatocytes. During tolerance and/or immune exhaustion, immune modulatory mechanisms result in dysfunctional T- or B-cells, but with intact T- (or B-) cell receptor on the surface [103]. 3) Chronic or persistent HCV infection **(Figure 5C)**; can occur when T-cells are not fully differentiated into functional effector cells, late generation of neutralizing antibodies or immune exhaustion. 4) Hypothetical protection from chronic HCV infection by vaccine-induced immune responses **(Figure 5D)**; vaccine-induced neutralizing antibodies, prevent infection while functional HCV specific T-cells protect from chronic infection.

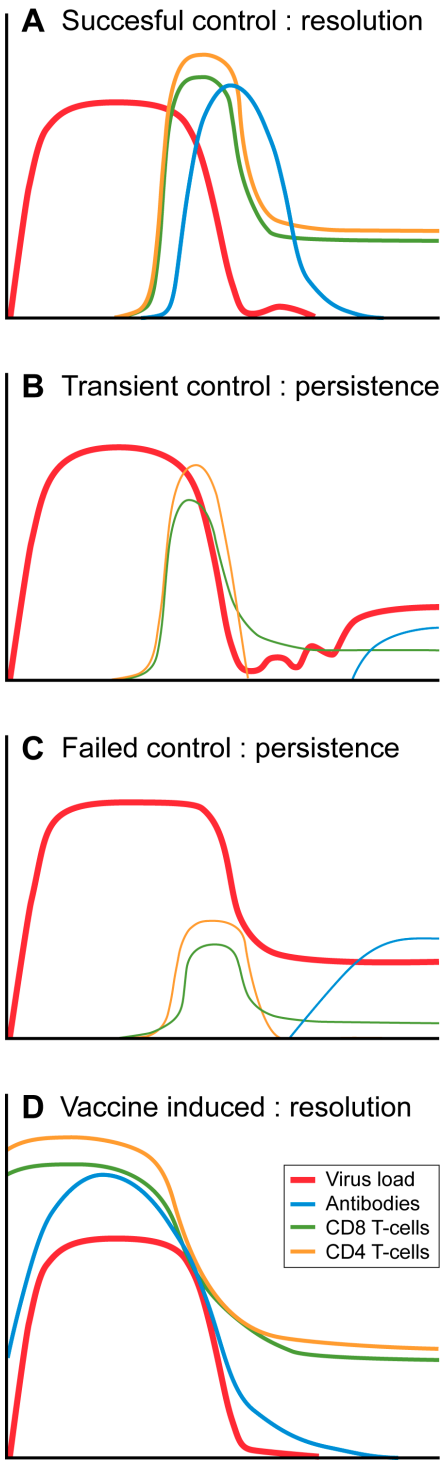

Figure 5; Schematic overview of the kinetics of the adaptive immune responses during HCV infection.

A) Viral clearance. Viral RNA (red line) is normally detected in blood within 1-2 weeks after exposure. The virus load will increase until the emergence of HCV specific CD4 (yellow line) and CD8 T-cell (green line) responses 4 to 8 weeks after infection [96]. Ideally, strain specific neutralizing antibodies (blue line) are present around the same time [120, 121]. After viral elimination, antibody responses can either remain present or decrease to undetectable levels. Memory T-cells remain usually present and can be detected by *in vitro* assays B) Transient control. After the initial peak viremia (red line), T-cell responses emerge and virus load decreases but remains detectable in serum. CD8 T-cells (green line) remain detectable but CD4 T-cell (yellow line) responses decrease to low levels. There appears to be a constant battle between virus and the immune system. *De novo* escape variants are able to evade the T and B-cell responses but at the same time lose viral fitness. When effective T and B-cell responses contract because the correct epitopes are no longer present, the virus "mutates back" to a more fit variant and virus load may increase again. Thinner lines of the adaptive immune responses represent decreased functionality of CD4 (yellow), CD8 T-cell (green) and antibodies (blue). C) Failed control leading to persistent infection; After the initial peak viremia, T-cell responses emerge and virus load decrease to lower levels but virus remains detectable in serum. T and B cells are functionally impaired or present in too low numbers to efficiently eliminate the virus. The virus remains present at steady state levels. Thinner lines of the adaptive immune responses represent decreased functionality of CD4 (yellow), CD8 T-cell (green) and antibodies (blue). D) Vaccine induced protection model. Vaccine-induced broadly neutralizing antibodies are present at the time of exposure and prevent virus production by infected hepatocytes. The hepatocytes that are infected are successfully eliminated by cytolytic T-cells in the liver.

Virus neutralizing antibodies

Already in 1994, it was described that plasma components had an important role in protection of HCV infection [104]. In a hallmark experiment by Farci *et. al.* [104], *in vitro* neutralizing capacity was determined by mixing infectious virus with heat inactivated plasma from the same patient and subsequently testing it for residual infectivity by inoculating the mixture into a naïve chimpanzee. Patient plasma collected 2 years after infection was able to prevent infection of the chimpanzee, while plasma collected 13 years after infection was not. At that time, there was no *in vitro* system available to confirm the presence of neutralizing antibodies. However, simultaneous appearance of envelope HCV specific antibodies in circulation [21] and mutations in the HVR of E2 of the virus [41, 105-107] substantiated the involvement of antibodies. This experiment not only demonstrated the protective capacity of neutralizing antibodies, but also showed the flexibility of the virus to escape immune pressure through mutation.

HCV specific antibodies that are generated during the acute phase of the infection are mainly directed against linear epitopes within structural and non-structural viral proteins. By contrast, neutralizing antibodies have been mapped to conformational epitopes within the E1 and E2 envelope proteins [108-114]. While most neutralizing antibodies are strain specific [114-116], broadly neutralizing antibodies, antibodies that recognize epitopes that are highly conserved between HCV genotypes, have also been described for E2 [115, 117, 118].

Specific targets for receptor binding have been identified in E2: CD81, SRB1 and coreceptors [119]. Neutralizing antibodies directed against domain I and III of E2 interfere with binding to CD81, while neutralizing antibodies directed against HVR-1 disturb binding of E2 to SRB1.

In humans, early induction of strain specific neutralizing antibodies was found to be associated with spontaneous recovery [120, 121]. In most cases however, these antibodies are only generated during the chronic phase of the infection. At this stage, the virus has disseminated and viral clearance is more difficult to achieve. Nonetheless, these antibodies may exert immune pressure that could potentially lead to decreased viral fitness.

The paradigm that neutralizing antibodies in chimpanzees play a less prominent role compared to humans, is mostly based on data collected by Logvinoff *et. al.* [121]. In that experiment they used a molecular clone derived from patient H to infect chimpanzees. In chimpanzees, neutralizing antibodies were observed after 15 to 20 weeks. By contrast, in patient H these antibodies were already observed 7 weeks post infection. This experiment however did

not take into account that the virus may have adapted rapidly to its new host, and therefore was slightly different from the original H77 clone. This could have caused the delayed formation of H77-neutralizing antibodies. Better understanding of the kinetics and specificity of HCV neutralizing antibodies may eventually lead to improved vaccine candidates.

T-cell responses

Antibody-mediated depletion experiments in chimpanzees showed that depletion of CD8 T-cells caused prolonged virus replication, and HCV was only cleared after recovery of HCV-specific CD8 T-cells in the liver [46]. On the other hand, CD4 T-cells were required for a complete control of HCV replication.[122]. Similarly, the association between HLA-class I and II molecules and HCV clearance emphasizes the role of both CD8 and CD4 T-cells (reviewed in [123]). Dysfunctional CD8 or CD4 cells may lead to persistent HCV infection.

Apart from viral escape, ineffective T-cell responses can also be a result of exhaustion [124-126]. Exhausted T-cells are characterized by loss of CD127 expression, decreased cytokine production or increased expression of inhibitory markers, like PD-1 and CTLA-4 **(Figure 4)**. Moreover, negative immune modulators, like Tim-3, LAG-3, CD160 and 2B4, have been associated with exhausted HCV specific T-cells [124, 127, 128].

Also active suppression of HCV-specific T-cell responses by regulatory T-cells (Tregs) or immunosuppressive cytokines, like IL-10 and TGF-β, have been described [129]. The contribution of each of these immuno-regulatory mechanisms during HCV persistence varies between individual patients, but also synergistic effects were observed [130]. Blocking these inhibitory molecules, temporarily restored HCV specific T-cell responses and resulted in a transient drop in virus load [128, 131-133]. Combining the recovery of functional T-cells by blocking T-cell inhibition with a vaccine that boosts T-cell responses, may therefore be an interesting therapeutic vaccine approach.

Chimpanzees as a model for chronic HCV infection?

To date, no case has been reported of a chimpanzee that developed fibrosis as a result of HCV infection. It is believed that intrinsic differences in IFN pathways may be, at least partly, responsible for this difference. It is therefore that chimpanzees are not the favorable animal model to study HCV-related pathogenesis.

It is tempting to draw a comparison with HIV infection in chimpanzees. Although chimpanzees can be infected with HIV, they do not develop AIDS. Recent reports however demonstrated that chimpanzees show signs of

immune activation comparable to what is found in HIV infected humans.

In contrast to HCV, the origin of HIV is known. Via several cross-species transmissions, the simian variant of HIV, SIV, has adapted, and is now able to infect humans. The origin of HCV is currently not known [134]. Yet, HCV-homologues have been isolated from horses and possibly dogs [135, 136] but clinical manifestations are very different from HCV infections in humans. In addition, equidistant hepaciviruses have been described in rodent species [137]. So far, only one HCV-like virus was isolated from a non-human primate. GBV-B was first described in a laboratory-housed new world monkey, but the ultimate origin of this virus unknown [134, 138]. Still, GBV-B is the only other hepacivirus that is known to induce immune-mediated pathology in the liver of a non-human primate. Persistent GBV-B infection is rare in a laboratory setting [139, 140].

To date, chimpanzees are still the only animal species in which persistent HCV infection can be studied. As discussed in detail in previous sections of this introduction, adaptive immune responses have been widely studied in order to dissect the differential immune responses between viral clearance and persistence. Although chimpanzees do not develop hepatic fibrosis, HCV infection in chimpanzees models non-progressive HCV infection in humans. Therefore, studying the consequences of chronic HCV infection in chimpanzees may contribute to improved understanding of HCV-induced disease and provide new insights necessary to develop and optimize therapeutic strategies.

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2

Protection From Chronic Infection:
The Role Of Vaccine-Induced Adaptive Immune Responses

2.1

Vaccine-Induced Early Control of Hepatitis C Virus Infection in Chimpanzees Fails to Impact on Hepatic PD-1 and Chronicity

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ABSTRACT

Broad T- and B-cell responses to multiple Hepatitis C Virus (HCV) antigens are observed early in individuals who control or clear HCV infection. The prevailing hypothesis has been that similar immune responses induced by prophylactic immunization would reduce acute virus replication and protect exposed individuals from chronic infection. Here we demonstrate that immunization of naïve chimpanzees with a multi-component HCV vaccine induced robust HCV-specific immune responses, and that all vaccinees exposed to heterologous chimpanzee-adapted HCV 1b J4 significantly reduced viral RNA in serum by 84%, and in liver by 99% as compared to controls (P=0.024 and 0.028 respectively). However, despite control of HCV in plasma and liver in the acute period, in the chronic phase three out of four vaccinated animals developed persistent infection.

Analysis of expression levels of proinflammatory cytokines in serial hepatic biopsies failed to reveal an association with vaccine outcome. However, expression of IDO, CTLA-4 [1] and PD-1 levels in liver correlated with clearance or chronicity. In conclusion, despite early control of virus load a virus associated tolerogenic-like state can develop in certain individuals independent of vaccination history.

INTRODUCTION

Hepatitis C virus (HCV) is the major cause of chronic liver infection leading to liver cirrhosis, with an increased risk of hepatocellular carcinoma [2]. Vaccines for the prevention of new HCV infections, or therapeutic immunization to facilitate viral clearance in the more than 170 million chronic carriers worldwide, would be of tremendous public health benefit [3]. Following a period of acute viremia and elevated serum ALT activity, approximately 70% of infected individuals develop persistent HCV infection leading to a slowly progressive chronic hepatitis with the subsequent sequelae [4]. The specific events leading to persistent HCV infection remain unclear, and it is apparent that there is a complex interaction between virus and host, in which both adaptive [5-11] and innate [12] responses [13] have been implicated, both influenced by the potentially tolerogenic liver environment [4, 14-16]. Analysis of peripheral blood samples and, where possible, of liver biopsies from both humans and chimpanzees has revealed immune correlates of viral clearance implicating an important role for non structural protein 3 (NS3)-specific cellular immune responses in the control and clearance of HCV infection [6, 14, 17, 18]. As prospective vaccine targets in addition to NS3, the more conserved core antigen, and the envelope glycoproteins E1 and E2 are potentially important, not only for protective T-cell immunity induced by the former [18-20], but also antibody responses against the envelope antigens [20-22], which may have neutralizing capacities able to control HCV viral loads as demonstrated recently in chimpanzees [11].

Here we test the hypothesis that vaccine induced rigorous and multi-specific B-, $CD4^+$ and $CD8^+$ T-cell responses would induce the control and consequently the clearance of HCV infection following exposure. As potential immune targets we utilized NS3 in combination of these three additional structural antigens in an HCV DNA prime, pox-virus boost vaccine strategy aimed at maximizing the breadth of adaptive B and T-cell responses.

MATERIALS & METHODS

Animals.

Six naïve mature chimpanzees (Pan troglodytes) were housed under conditions for optimal social and health care [23]. All vaccine trial procedures were critically assessed for ethical care and use in accordance with international guidelines. Blood sampling and liver biopsies were performed as previously described [18]. Body weight, temperature, hematology and biochemistry clinical values were monitored at regular monthly to bimonthly intervals**.**

HCV vaccine vectors.

Two DNA plasmids expressing the core-E1-E2 and NS3 of the HCV genotype 1b, J4 strain [24] were used for the priming immunizations. The NS3 encoding plasmid was described previously [25]. The CoreE1-E2 (aa 1-746) gene sequence was inserted into the gWiz plasmid (Gene Therapy System INC, San Diego) to create pgWizCE1E2 [25]. As the immunogenicity of NS3-4 encoding DNA plasmids in primates was found to be of no additional benefit over the NS3 DNA [26 and unpublished data], the NS3 encoding DNA plasmid was selected. The empty plasmid, pgWiz, was used to immunize control animals.

Recombinant Modified Vaccinia Ankara (MVA) expressing core-E1-E2 and NS3 gene sequences were constructed by transient host range selection [27-29] using the plasmids pCMV-C980 and pCMV-N-729-3010, encoding for HCV-1b [24] structural (aa 1-830) and NS3 (aa 1028-1658) proteins. Recombinant viruses were amplified and purified by ultracentrifugation through sucrose [29]. Non-recombinant MVA was used to immunize control animals.

Peptides and recombinant proteins.

Fifteen-mer peptides with overlaps of 7 amino acids covering the core, E1, E2 and NS3 sequences of genotype 1b, J4 strain [24] were purchased from Clonestar Biotech (Brno, Czech Republic). The peptides covering NS4 and NS5 proteins were 15-mers with 5 overlap, or 20-mer with 10 overlap for the aa positions 1875 to 2454, and were purchased from EMC microcollections GmbH (Tuebingen, Germany). Core polypeptide (aa 1-120 derived from HCV genotype 1a [30]) fused to a His-tag was expressed in *E. coli* strain BL21 (DE3), and purified under denaturing conditions on a Ni-NTA agarose column (Qiagen). Sequences of NS3 helicase (aa 1192-1457), E1 (aa 192-326), and

E2 (aa 384-673) were derived from the HCV-1b strain. His-tagged NS3 was expressed in *E. coli* JM109 and purified on a Ni-NTA column. E1 and E2, deleted for their transmembrane domain, were cloned into pT-alpha vector. After transfection of CHO DHFR- cells, E1 or E2 were purified from supernatant on Ni-NTA agarose column. Analysis of the eluted protein fractions was performed by SDS-PAGE and Coomassie blue staining.

Immunization and HCV exposure.

The animals were immunized at weeks 0 and 6 with DNA plasmids encoding core-E1-E2 (left arm) and NS3 (right arm) or pgWIZ (both arms) at a dose of 2 mg per inoculum, equally divided intramuscularly and intradermally. Booster immunizations were given at weeks 14 and 20 with MVA encoding core-E1-E2 and NS3 or wildtype ($1x10^9$ pfu per inoculum, at the corresponding locations). At week 28, eight weeks following the last immunization, all animals were challenged intravenously with 25 CID_{50} of *in vivo* titrated HCV 1b J4 (generously provided by Robert H. Purcell, NIAID, NIH, Bethesda, MD), diluted in autologous pre-immune plasma. The vaccine and challenge HCV strains were both genotype 1b, differing in approximately 5 % of total amino acids.

Humoral immune responses.

Anti-HCV antibody responses in sera were measured using microplate wells coated with HCV core (0.5 mg/ml), E1 (4 µg/ml), E2 (1 µg/ml) or NS3-helicase proteins (0.5 mg/ml). ELISA was performed as described previously (Komurian-Pradel et al., in press). The capacity of the chimpanzee sera to neutralize HCV was analyzed using HCV pseudo-particles in infection assays on HuH-7 target cells as previously described [31]. HCV pseudoparticles were generated as previously described using expression vectors encoding the viral components including E1E2 glycoproteins of strain CG1b [32]. Control neutralizations were performed using pseudo-particles generated with glycoproteins derived from the feline endogenous retrovirus RD114 (RD114pp).

C**ellular immune responses.**

HCV-specific lymphoproliferation was determined with peripheral blood mononuclear cells (PBMC) as described previously [18]. Quantification of specific cytokine secreting cells was performed using IFNγ, interleukin 2 (IL-2) and interleukin 4 (IL-4) enzyme-linked immunospot (ELISPOT) assays according to the manufacturer's instructions (U-Cytech, Utrecht, Netherlands)

and as described previously [18], using the core, E2 and NS3 recombinant proteins, and peptide pools covering E1 and NS3. Assays were performed in triplicate. An analysis of variance was performed on the log_{10}-transformed data for each chimpanzee at each time-point. Means of antigen-stimulated wells were compared to the medium-alone wells using a Studentized range test. ICS was performed as described previously [33] by stimulating PBMCs ($5x10^6$/ml) with either Con-A, peptide pools (5 µg/ml of each peptide) or medium alone, and staining with FITC labeled anti-CD3 and PerCP labeled anti-CD8, PE labeled anti-IL-2 and APC labeled anti-IFNγ mAb (BD Pharmingen), allowing proper discrimination between $CD3^+$ $CD8^+$ responding cells from all other $CD3^+$ cells The data obtained from the ICS assay were analyzed by using a test for differences between ratios, comparing the number of cytokine positive cells in the presence of the HCV antigen to the amount of cytokine positive cells in the medium control, with a two tailed test and alpha = 0.05. Only results with statistical significance are shown.

Virus quantification and sequencing.

For serum samples, HCV 5'NTR sequences were detected in a non-quantitative nested real time polymerase chain reaction (RT-PCR) essentially as described by Krajden *et al*. [34]. Independently, in another laboratory, all serum samples were quantified blindly for HCV RNA: RNA was extracted with the Nucleospin kit (Macherey Nagel) and eluted in RNAase free water. Sera were tested for a quantitative HCV RNA by real-time polymerase chain reaction (PCR) of the 5' HCV non-coding region [35, 36]. In addition, several serum samples were also blindly quantified for HCV RNA in a third laboratory, using the COBAS AMPLICOR™ HCV MONITOR test (Roche Molecular Diagnostics) (data not shown). For liver biopsies, frozen tissue was disrupted and RNA extracted with the High Pure RNA Tissue kit (Roche). HCV RNA-positive and -negative strand quantification in liver biopsies was carried out blindly by real-time PCR using molecular beacon detection [37]. The NS3 region was targeted for amplification by nested-PCR. PCR products were directly sequenced by using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystem) and ABI PRISM 3100 genetic analyzer (PE/Applied Biosystem). Sequence analysis was carried out with Vector NTI suite (Invitrogen).

Quantification of expression of TGF-β, IFN-α, CD4, CD8, IL-10, IL-5, Interferon (IFN)γ, TNFα and CCR7 from liver tissue was performed by real-time RT-PCR. RNA was extracted from the liver biopsies using the Stratagene Absolutely RNA RT-PCR miniprep kit. Real-time PCR was performed in SYBR Green PCR master mix (Applied Biosystems) containing human cDNA for

positive controls, for 40 cycles using an ABI 7700 Prism. Expression of PD-1, Foxp3, CTLA-4, IDO and Cox2 mRNA in liver was quantified as described [1].

Statistical analysis.

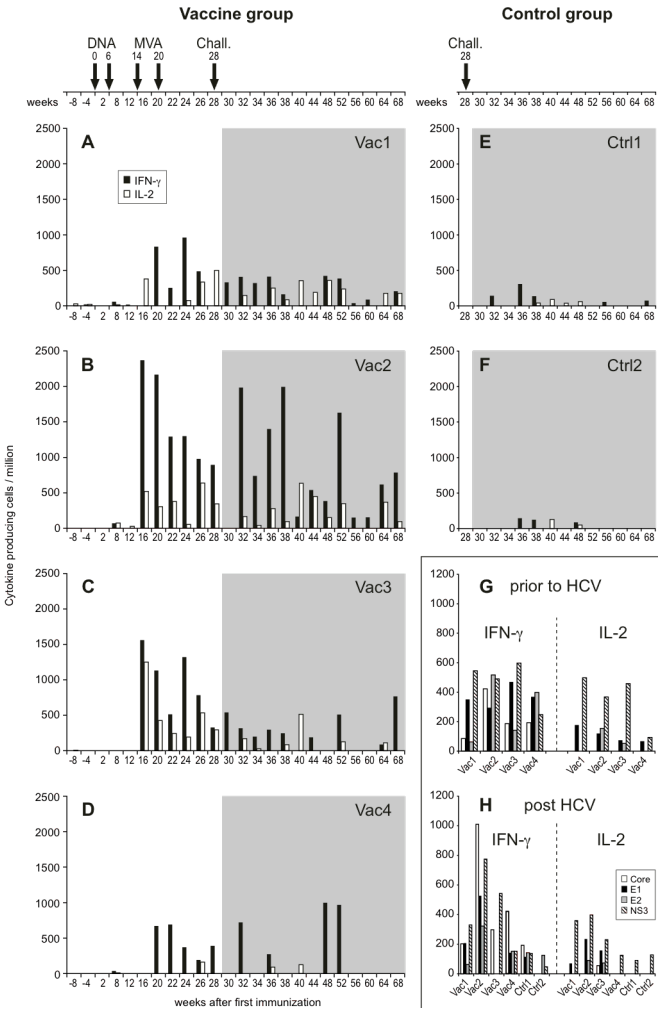
To compare the serum and liver HCV RNA in the four vaccinated versus the two control animal, two-tailed Student's t-tests were performed on $\log_{10}$-transformed data to ensure normality, with alpha = 0.05, and P values calculated by exact methods, and given to two significant figures. When a sample was negative, a value of 1 (serum HCV RNA) or the sensitivity of the assay (liver HCV RNA) was assigned for facilitating data analysis.

RESULTS

Induction of HCV-specific Th1 and Th2-cytokine responses

DNA prime – MVA boost immunization induced strong Th1-cytokine responses against multiple antigens in all four vaccinated animals as determined by ELISPOT **(Figure 1A-D)**. As expected, DNA priming induced low to undetectable HCV antigen specific responses, not exceeding 73 cytokine-producing cells / 10^6 PBMCs. However, strong immune responses were induced after the first MVA boost immunization, reaching 2368 IFNγ producing cells / 10^6 PBMCs (Vac2), and 1250 IL-2 producing cells / 10^6 PBMCs (Vac3). The IFNγ responses were 2 to 4 times higher than the IL-2 in Vac1, 2 and 3, while Vac4 elicited virtually no IL-2 responses. The analysis of individual responses to each vaccine antigen after the course of immunization showed that the IFNγ responses targeted all four vaccine antigens in each vaccinee, NS3 and E1 being consistently high, while strong IL-2 response were observed only against NS3 **(Figure 1G)**.

Figure 1; Time course and magnitude of HCV-specific IFNγ and IL-2 T-cell responses by ELISPOT during immunization and after HCV challenge.



(A-F) Vaccination and challenge schedule is shown at the top. Arrows correspond to the time-points of each immunization and to the HCV 1b J4 challenge. Shaded areas represent time-points from the challenge date onwards. Total response was calculated by summing responses against each HCV antigen (core recombinant protein, E1 peptide pool, E2 and NS3 recombinant proteins). (G and H) Individual HCV-specific cytokine responses to the four vaccine antigens: (G) prior to challenge (weeks 22-28), and (H) post HCV exposure (weeks 2-12 post exposure, acute phase). The exact time points at which high responses were reached to each antigen could differ from 2 to 6 weeks between animals, therefore the highest response for each animal and each antigen over the indicated period is displayed.

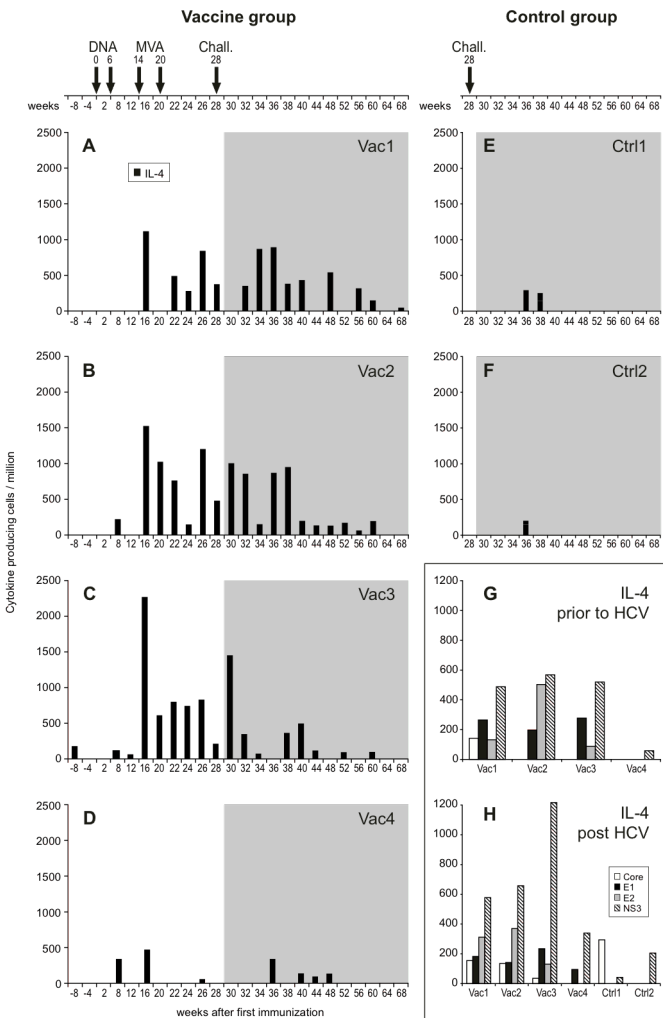


Figure 2; Time course and magnitude of HCV-specific IL-4 T-cell responses by ELISPOT during immunization and after HCV challenge.

(A-F) The vaccination and challenge schedule is shown at the top. Arrows correspond to the time-points of each immunization and to the HCV 1b J4 challenge. Shaded areas represent time-points from the challenge date onwards. Total response was calculated by summing responses against each HCV antigen (G and H). Individual HCV-specific IL-4 responses to the four vaccine antigens: (G) prior to challenge(weeks 22-28), and (H) post HCV exposure (weeks 2-12). The exact time points at which high responses were reached to each antigen could differ from 2 to 6 weeks between animals, therefore the highest response for each animal and each antigen over the indicated period is displayed

The induction of Th-2 type T-cell responses was assessed by measuring the IL-4 production in ELISPOT assays. Strong IL-4 responses were induced in Vac1, 2 and 3, ranging from 1115 (Vac1) to 2267 (Vac3) IL-4 producing cells /10^6 PBMCs after one MVA administration **(Figure 2A-D)**. In Vac4 however, the IL-4 response was lower, reaching only 473 IL-4 producing cells / 10^6 PBMCs after the first MVA injection, and was not sustained. Similar to the IL-2 responses, the IL-4 responses targeted mainly NS3, with 58 to 568 IL-4 producing cells / 10^6 PBMCs prior to HCV exposure **(Figure 2G)**.

We also assessed the proliferative capacity of the HCV-specific T-cell responses **(Figure 3)**. All vaccines elicited HCV-specific lymphoproliferative responses after DNA and MVA administration, Vac2 eliciting the highest response **(Figure 3A-D)**. However, the responses observed in Vac3 and Vac4 were transient, suggesting that the vaccine elicited poor T-cell memory in these two vaccinees. The antigen targeted by the vaccine was predominantly NS3 in all four animals **(Figure 3G).**

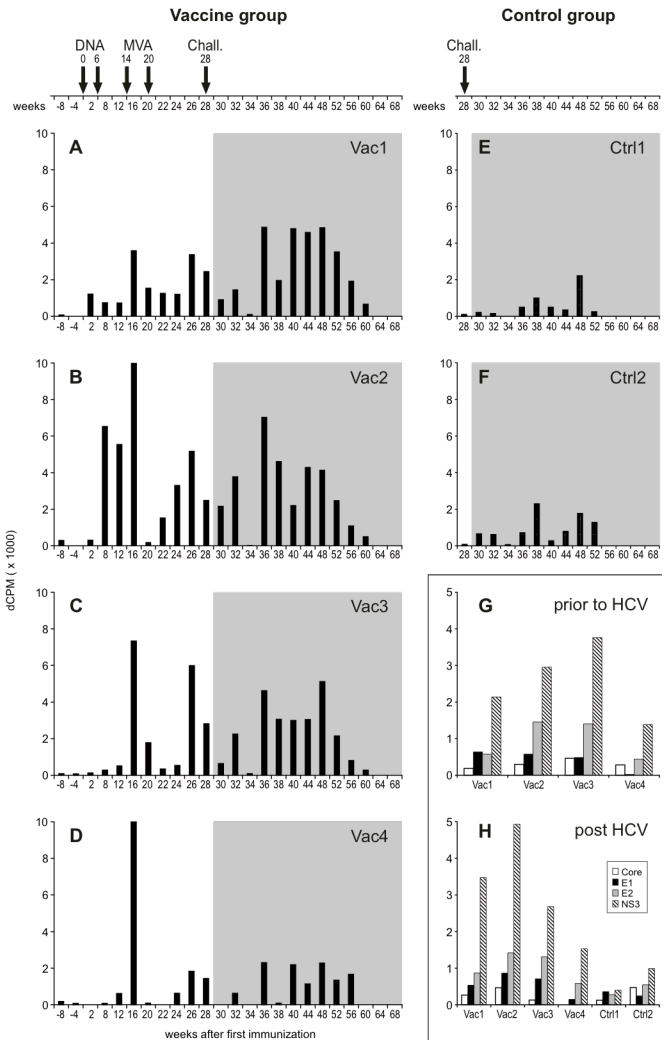


Figure 3; Time course and magnitude of HCV-specific lymphoproliferative T-cell responses during immunization and after HCV challenge.

(A-F) The vaccination and challenge schedule is shown at the top. Arrows correspond to the time-points of each immunization and to the HCV 1b J4 challenge. Shaded areas represent time-points from the challenge date onwards. Total response was calculated by summing responses against each HCV antigen (G and H). Individual HCV-specific lymphoproliferation to the four vaccine antigens: (G) prior to challenge (weeks 22-28), and (H) post HCV exposure (weeks 2-12). The exact time points at which high responses were reached to each antigen could differ from 2 to 6 weeks between animals, therefore the highest response for each animal and each antigen over the indicated period is displayed

Induction of HCV-specific CD8 T-cell responses

The production of IFNγ in the ELISPOT assay to specific HCV with peptide pools provided first indication of the presence of HCV-specific $CD8^+$ T cells. Our results showed that three out of four vaccinees had elicited strong NS3 peptide-specific IFNγ responses at two weeks after the first MVA injection, reaching up to 1785 IFNγ producing cells / 10^6 PBMCs in Vac3, while the IFNγ production in response to E1 peptides remained modest **(Figure 4A-C)**. Only Vac4 elicited a poor IFNγ response to the E1 and NS3 peptides **(Figure 4D)**. The presence of $CD8^+$ T-cell responses was confirmed using ICS with overlapping peptide pools covering the four antigens encoded in the vaccine. At week 22, $CD8^+$ IFNγ responses to NS3 were confirmed in Vac1 and Vac3, reaching 0.16% of $CD8^+$ cells in Vac1 **(Figure 4G)**. This represented 323 IFNγ producing $CD8^+$ cells / 10^6 lymphocytes, directly suggesting that the marked IFNγ production in the ELISPOT assay was largely generated by $CD4^+$ T-cells.

These results showed that all vaccinees elicited consistent multi-antigen and multi-functional specific T-cell responses as indicated by high numbers of cytokine producing cells, with a cytokine bias indicative of Th0-like responses, predominantly focused on NS3.

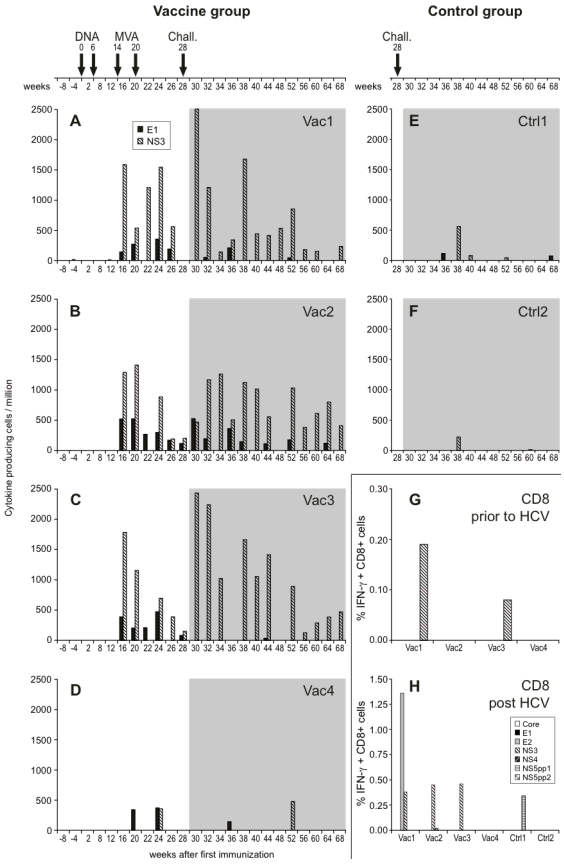


Figure 4; Time course and magnitude of E1 and NS3 peptide pool-specific IFN-γ T-cell responses by ELISPOT and ICS during immunization and after HCV challenge.

(A-F) The vaccination and challenge schedule is shown at the top. Arrows correspond to the time-points of each immunization and to the HCV 1b J4 challenge. Shaded areas represent time-points from the challenge date onwards. The response to E1 was obtained by using one peptide pool covering the E1 sequence, and the total NS3 response was calculated by summing responses against the two peptide pools covering aa 1028 to 1346 and aa 1340 to 1659 respectively (G and H). The individual HCV peptide-specific percentages of IFN-γ^+, $CD8^+$ responses were obtained by intracellular cytokine staining at the indicated time-points: (G) prior to challenge (week 22), and (H) after HCV exposure (week 34).

Vaccine-induced HCV-specific B-cell responses

The B-cell responses were analyzed by ELISA and by neutralization assays. The DNA prime alone was a poor inducer of antibody responses, but the prime-boost combination elicited high HCV-specific antibody titers in all four animals, peaking between 1,300 and 64,100 after the first MVA administration, but with no further increase after the second MVA immunization **(Figure 5A)**. Although most of the antibody responses targeted as desired the envelope proteins E1 and E2 before **(Figure 5B)**, or after challenge **(Figure 5C)**, no neutralizing activity was detected in an HCV 1b pseudoparticle assay, either prior to, or after HCV challenge (data not shown).

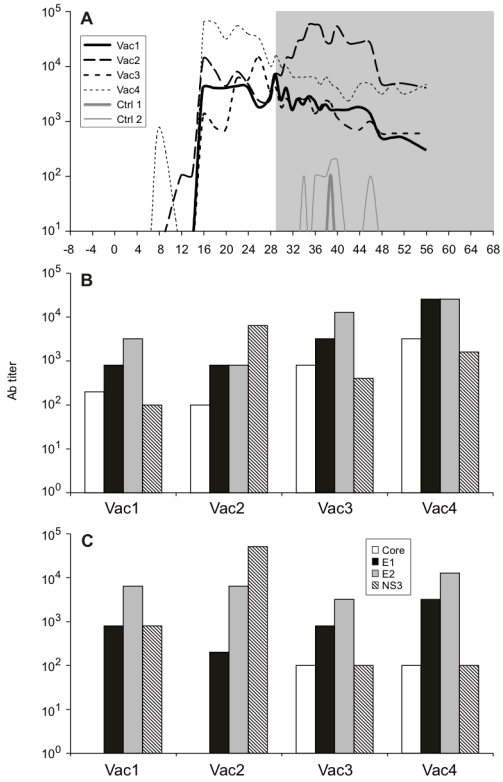


Figure 5; Time course and magnitude of HCV-specific antibody responses during immunization and after HCV challenge. (A) The vaccination and challenge schedule is shown at the top. Arrows correspond to the time-points of each immunization and to the HCV 1b J4 challenge. Shaded areas represent time-points from the challenge date onwards. Total response was calculated by summing responses against each HCV antigen (core, E1, E2 and NS3). (B and C) Individual HCV-specific antibody titers to the four vaccine antigens: (B) and post HCV challenge (weeks 2-12) (C) are represented. The exact time points at which high responses were reached to each antigen could differ from 2 to 6 weeks between animals, therefore the highest response for each animal and each antigen over the indicated period is displayed

Vaccine-induced control of early virus load

Eight weeks following the last immunization, all animals were challenged with HCV 1b J4. Over the first three months of follow up, the two vector control chimpanzees (Ctrl1 and Ctrl2) developed HCV viremia with peaks of serum HCV RNA of 778,125 and 766,000 copies / ml respectively **(Figure 6A and B)**. Positive strand HCV RNA was detected in the liver (8,990 and 3,550 copies / µg of total RNA), as well as minus-strand RNA, with positive / minus strand ratios of 1.4:1 and 2.7:1. In contrast to the control animals, all immunized animals maintained markedly lower virus loads in serum during the acute phase, as observed by the 74% reduction of peak viremia (P=0.030, peaks of 314,500 in Vac1, 74,775 in Vac2, 307,375 in Vac3 and 242,250 copies/ml in Vac4, **Figure 7A**), and the 84% reduction in total serum viral loads during the first three months follow up (P=0.024 **Figure 7B**). Quantitation of HCV loads in the liver four weeks post exposure provided additional evidence that HCV replication was greatly reduced in all vaccinated animals. Positive HCV RNA strand production was reduced by at least 99% in the vaccinated group as compared to the controls (P=0.028). Moreover, HCV minus strand RNA was undetectable in all vaccinees (>99.7% reduction,

P=0.0003, **Figure 6**). These results demonstrate that HCV-specific vaccine-induced adaptive immune responses controlled and markedly limited HCV replication in the first three months of infection. The most striking effect was observed in Vac2, whose peak viremia never exceeded 74,775 RNA copies/ml of plasma.

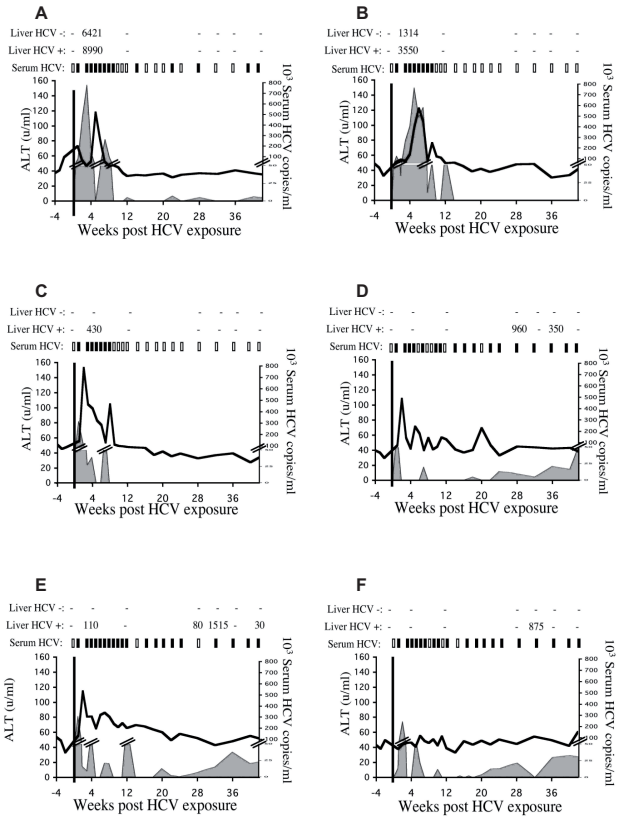


Figure 6. Course of infection in control chimpanzees.
Ctrl1 (A) and Ctrl2 (B), and in vaccinated chimpanzees Vac1 (C), Vac2 (D), Vac3 (E) and Vac4 (F). All animals were challenged at week 28 following immunization (dashed vertical line). The liver enzyme ALT levels are represented as black lines, left Y-axis. Quantitative HCV-RNA levels are shown as shaded areas, right Y-axis. Nested HCV RNA PCR results on serum are indicated above each graph as: PCR □: negative and ▮: positive. Quantification of HCV RNA in liver was performed prior to HCV exposure and at weeks 4, 12, 28, 32, 36 and 42 post exposure. Levels of HCV positive strand (Liver HCV +) and negative strand (Liver HCV –) are expressed as copies / µg total RNA, with - meaning below detection

Absence of protection against chronic infection

The impact of immunization on the control of HCV virus load was observed in all four vaccinees during the period of primary viremia. However, these effects on viremia did not extend into the chronic phase when viral clearance in the vaccinated animals was anticipated. Ultimately, only one vaccinee (Vac1) successfully cleared HCV infection **(Figure 6C)**. In contrast to Vac1, the three other immunized chimpanzees failed to clear the virus in spite of a markedly lower peak viremia than controls. Vac3 remained HCV-positive at most time-points during follow-up **(Figure 6E)**. Although no viral RNA was detected in the plasma of the remaining two immunized animals (Vac2 and Vac4) between weeks 9 and 18 post exposure using the

quantitative assay (detection limit 500 copies/ml), HCV RNA could be detected at several time-points **(Figure 6D and F)**. In addition, by week 20 post HCV exposure, viral RNA could again be quantitated at several time-points in Vac2, Vac3 and Vac4, with titers ranging from 500 to 22,302 RNA copies / ml of plasma. Results were confirmed by an independent quantitative HCV RNA assay performed on other serum aliquots (COBAS, data not shown). No minus strand HCV RNA was detectable in liver biopsies from all vaccinees taken at weeks 28, 32, 36 and 42 post exposure, but positive strand RNA remained present in three of the four animals (Vac2, Vac3 and Vac4, **Figure 6A-F**). Different patterns were also observed between controls in the acute versus the chronic phase. While virus loads of the control animals during the acute viremic phase were markedly greater than those in the vaccinees, this was not the case in the chronic phase. Of the two control animals, Ctrl1 remained persistently infected, whereas in Ctrl2 HCV RNA levels declined and became negative by all assays **(Figure 6A and B)**, suggesting that mechanisms other than adaptive immune responses contributed to viral clearance. Indeed, post HCV exposure both control animals developed weak T-cell immune responses to HCV, which decreased rapidly over time **(Figures 1, 2 and 3)**. Specific $CD8^+$ responses could not be detected in Ctrl2 while Ctrl1 elicited a $CD8^+$ response to NS5 pp1 **(Figure 4H)**. Immune responses induced by immunization were predominantly directed to NS3 and were associated with early control of infection in all four vaccinees, though subsequently only transient control was achieved in three animals. The animal that sustained complete viral control and clearance, Vac1, did generate multi-

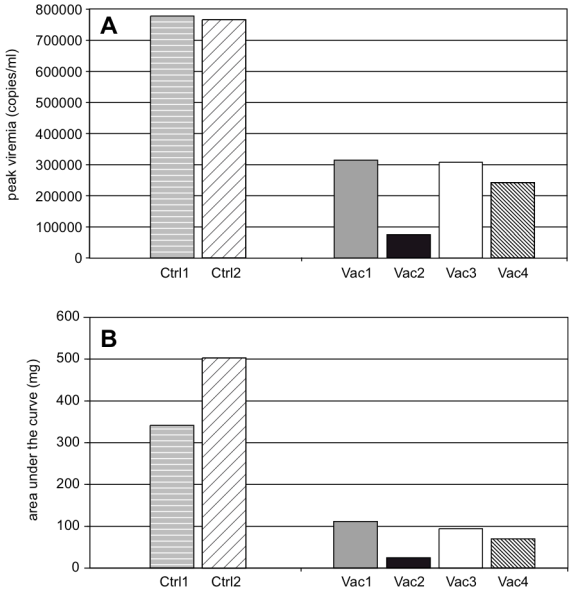


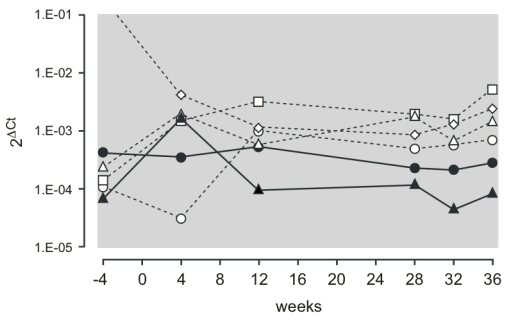
Figure 7. Differences in virus loads in control versus vaccinated animals based on two different parameters

A) a 74% reduction of acute phase peak viremia is observed when individual peak virus concentrations are compared, left two bars represent values in unvaccinated controls, right, bars corresponding to the four vaccinated animals. B) compiled area under the curve reveals a reduction of 84% in virus load in the four vaccinated animals.

functional T-cell responses (IFNγ and IL-2 and IL-4) to multiple HCV antigens. However, these were not necessarily of greater magnitude prior to, nor following HCV exposure compared to the other three vaccinees **(Figures 1, 2 and 3)**. However, Vac1 did elicit stronger $CD8^+$ responses to NS3 prior to challenge (0.19% of $CD8^+$ lymphocytes). Additionally, this animal mounted a strong $CD8^+$ response to E2, peaking at 1.36% of $CD8^+$ cells six weeks post exposure, **(Figure 4H)**. These responses eventually decreased in Vac1 over time indicative of successful clearance, while Vac2 and Vac3 subsequently developed detectable NS3-specific $CD8^+$ responses (from 0.45 to 0.77% of IFNγ secreting $CD8^+$ T cells, **Figure 4H**) that were maintained up to the end of the follow up (data not shown).

To investigate underlying causes of the discordance between the vaccine induced control in the acute phase, to the unpredicted causes of chronic persistence, we undertook an analysis of cytokines and immune regulatory molecules in hepatic tissue, Comparison between animals which cleared HCV with those which became persistently infected revealed no significant differences in the expression of TGF-β, IFNα, CD4 and CD8, IL-10, IL-5, IFNγ, TNFα, CCR7, FoxP3, CTLA-4 and Cox2 in liver biopsies before and after HCV challenge (data not shown). Strikingly, despite fluctuations in the acute phase, by week 12 post infection a consistent correlation of higher PD-1 expression in hepatic tissues was observed in animals which developed persistent infection versus lower levels in those which resolved infection (Vac1 and Ctl2, **Figure 8**). Not only was this effect observed with PD-1 but also with levels of expression of IDO and CTLA-4 (data not shown [1]), There is a close interaction between CTLA-4 and IDO, given CTLA-4's ability to stimulate IDO activity which ultimately favors development of tolerogenic dendritic cells [38]

Figure 8. Expression of PD-1 in hepatic tissues over time.



Quantitative PCR levels of PD-1 from longitudinal liver biopsies from each of the study animals, from 4 weeks prior to infection, to 36 weeks, post-infection. All four animals which became chronically infected (open symbols) have elevated levels of PD-1 from week 12 onwards. (-△- = Vac 2, -□- = Vac 3, -◇- = Vac 4, -○- = Ctrl1) in contrast to lower levels in animals which cleared HCV infection (-▲- = Vac 1, -● = Ctrl 2). Data presented relative to actin (not shown) and CD4 (above) show similar kinetics over time.

DISCUSSION

With world-wide estimates of more than 170 million infected humans, chronically carrying HCV, the potential risk of transmission is great and the need for a prophylactic vaccine is apparent. Here, we report on a prophylactic HCV vaccine study aimed at inducing immune responses capable of (i) reducing acute phase viral load, and (ii) subsequently preventing persistent infection, and thus chronic liver disease. For this study we used a heterologous HCV 1b J4 isolate that has previously established chronic infection in all 7 naïve chimpanzees in which it had been used [18, 39-41]. Following immunization we demonstrated that robust vaccine induced immune responses to multiple HCV antigens were possible. Upon exposure to the heterologous 1b strain there was a dramatic reduction of HCV replication in the early phase in all vaccinated individuals *in vivo.* Similar results were observed recently with a T-cell based vaccine against a 1a challenge [8]. However, based on the characteristics of this 1b genotype to induce a high prevalence of persistent infection, we were able to demonstrate that the transition to chronic phase HCV viremia is not directly linked to early control.

Early peak viral titers are a characteristic of this inoculum, occurring between 1 to 2.5 weeks post exposure [18, 39, 40]. It has been suggested that T-cell responses must be sustained for weeks or months beyond the point of apparent control of virus replication to prevent relapse and establishment of persistent infection [42, 43]. In the absence of data concerning HCV-specific immune responses of vaccinated animals that were not exposed to HCV, we can only speculate that the relatively rapid decline of the HCV-specific immune responses post-infection may be due to a specific immune impairment by the virus itself. Indeed, impairment of adaptive immune responses in the presence of HCV infection in humans has been reported at both the level of dendritic and T-helper cells [44-47]. Recently, several studies have reported that T-helper dependent $CD8^+$ T-cell effector function is reduced in HCV infection (reduced proliferation, perforin and IFNγ expression), similar to observations in HIV infected individuals [48-52]. In addition, while strong HCV specific humoral and $CD4^+$ T-cell responses were induced in all vaccinees, vaccine-induced $CD8^+$ T-cell responses detected by intracellular cytokine staining were rather weak in three of the four vaccinees. The strongest response was observed in the vaccinee with sustained viral clearance. In an effort to understand potential local and limiting effects in the

liver we measured mRNA expression of IFNγ, IL-5, IL-10, TNFα, CCR7 and TGF-b in hepatic biopsies of all study animals. This panel of cytokines unfortunately failed to reveal any correlation with effective viral clearance or persistence.

There was little or no boost of the T-cell responses in the vaccinated chimpanzees following the second MVA boost or challenge. Over-stimulation or exhaustion of T-cell responses by MVA booster immunization seems only likely in the case of Vac4, as in this particular animal high levels of non-HCV background IFNγ were induced following MVA boosting which were maintained until shortly before challenge.

Following acute HCV infection T-cell responses became poorly functional particularly in individuals which became chronically infected, which is reminiscent of immune exhaustion. Longitudinal liver biopsies revealed higher PD-1 expression in relationship to actin as well as CD4 expression in the four animals which became chronically infected in contrast to the lower levels in the two animals which cleared infection. These findings are supported by recent observations of elevated PD-1 expression in HCV infected persons [53] in which PD-1/PDL-1 receptor ligand blocking was reported to improve function of HCV specific CD8 T-cells. Our findings of elevated PD-1 in hepatic tissue provide mechanistic insight into HCV persistence as well as possible therapeutic and vaccine strategies. Findings of elevated levels of IDO and CTLA-4 expression from the same biopsies provided additional insights into underlying mechanisms for this impaired immune function [1]. Increased IDO provokes tolerogenic-like effects when induced by pro-inflammatory cytokines such as IFNγ and TNFα [1, 38, 54]. Both lines of evidence point towards the risk of driving an over zealous IFNγ $CD8^+$ T-cell response that in particular may be counter productive if directed to promiscuous HCV epitopes that fail to impact sufficiently on viral fitness. Such rigorous but largely ineffective T-cell responses may in fact drive or compound a tolerogenic environment in the liver. Detailed follow-up studies are currently underway to characterize the conserved or promiscuous nature of epitope-specific $CD4^+$ and $CD8^+$ T-cell responses and their MHC – restricting molecules in these animals.

The data presented here clearly demonstrate the potential of HCV vaccine candidates to elicit host immune responses capable of controlling HCV 1b viremia both in plasma as well as liver in the acute phase of infection. While these are important proof of principle findings with respect to HCV

vaccine development, they also point to the challenges ahead. Identifying conserved vaccine targets for CTL based vaccines that will provide efficacy in a large outbred population is a tall order. This is underscored by a cluster of recent reports [5, 9, 10] highlighting the potential for CTL escape by HCV in infected human cohorts [55]. These data, together with growing evidence of HCV induced tolerance in hepatic sites provides new insight into the challenges, as well as potential strategies, for the induction of effective immunity to HCV.

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2.2

Strong Vaccine-Induced CD8 T-cell Responses Have Cytolytic Function In A Chimpanzee Clearing HCV Infection.

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ABSTRACT

A single correlate of effective vaccine protection against chronic HCV infection has yet to be defined. In this study, we analyzed T-cell responses in four chimpanzees, immunized with core-E1-E2-NS3 and subsequently infected with HCV1b. Viral clearance was observed in one animal, while the other three became chronically infected. In the animal that cleared infection, NS3-specific CD8 T-cell responses were observed to be more potent in terms of frequency and polyfunctionality of cytokine producing cells. Unique to this animal was the presence of killing-competent CD8 T-cells, specific for $NS3_{1258\text{-}1272}$, being presented by the chimpanzee MHC class I molecule Patr-A*03:01, and a high affinity recognition of this epitope. In the animals that became chronically infected, T-cells were able to produce cytokines against the same peptide but no cytolysis could be detected.

In conclusion, in the animal that was able to clear HCV infection not only cytokine production was observed but also cytolytic potential against specific MHC class I/peptide-combinations.

INTRODUCTION

Hepatitis C virus (HCV) infection is characterized by a high propensity for development of chronic infection, which typically manifests as asymptomatic for a long period of time. However, over decades the virus causes subtle, but cumulatively irreversible, hepatic damage. A vaccine that could prevent development of persistent HCV infection would therefore be of great clinical benefit.

The chimpanzee is the only validated animal model of HCV infection [1, 2]. The dilemma that biomedical research in non-human primates, and chimpanzees in particular, is inevitably associated with low animal numbers and limiting statistical analysis, has been discussed elsewhere [3]. Nevertheless, we believe that results from in depth immune-profiling of immunized chimpanzees may provide new insights into immune mechanisms operating in the early phase after infection and as such is important for optimal vaccine development in the future.

Spontaneous HCV clearance has been associated with the presence of broad and strong T-cell responses in both humans and chimpanzees [4-7]. Moreover, specific memory T-cell responses often correlate with early clearance after HCV reinfection [8-10]. These data imply that T-cell-based vaccines may facilitate HCV clearance, protect from viral persistence and thus from HCV-related disease progression. For this reason, several HCV T-cell vaccine-candidates have been evaluated. Some of these vaccine-strategies induced strong T-cell responses however, no correlation was observed between either the magnitude or the breadth of vaccine-induced T-cell responses, and viral clearance. The lytic capacity of vaccine-induced CD8 T-cells, in the context of MHC class-I presentation was not previously evaluated in relation to viral clearance.

Previously, we reported partial control of an HCV 1b challenge after a DNA prime-MVA-boost vaccine strategy, targeting HCV core, E1, E2 and NS3 in chimpanzees [11]. In these animals, neither (neutralizing) antibody responses nor *ex vivo* cytokine production or proliferative responses were uniquely associated with control of HCV infection. Therefore, the current study aimed to identify potential mechanisms of protection via detailed functional characterization of the T-cell responses against the most dominantly

recognized antigen, NS3 [11].We report that the animal that cleared infection had a high percentage of polyfunctional cytokine producing CD4 and CD8 T-cells against NS3-peptides. Moreover, a strong cytolytic T-cell (CTL) response against epitope $NS3_{1258-1272}$ was identified, which was uniquely presented in the context of the chimpanzee MHC class I molecule Patr-A*03:01.

MATERIALS & METHODS

Ethics statement

This retrospective study was performed on cryopreserved PBMCs, isolated from animals that were part of an HCV vaccine-efficacy study [11]. As previously described, the preceding study had been performed in six purpose bred, naive mature chimpanzees (*Pan troglodytes*) that were housed at the Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands, according to international guidelines for non-human primate care and use (The European Council Directive 86/609/EEC, and Convention ETS 123, including the revised Appendix A). The Institutional Animals Care and Use Committee (DEC-BPRC) approved study protocols (#426) according to strict international ethical and scientific standards and guidelines. The qualification of the members of this committee, including their independence from a research institute, is stated in the "Wet op de Dierproeven" (1996). The experiment can only be performed after a positive recommendation from this ethical committee. Permission for projects on non-human primates is requested in an animal ethical committee form. This form is discussed in the ethical committee and, if approved, a written permission is sent to the project leader and a copy to the director of the BPRC, who holds licence to perform animal experimentation. The project was monitored by a qualified independent veterinarian, specifically regarding the ethical issues of the projects.

The animals were in good physical health with normal biochemical and hematological values. The animals were socially housed in a BSL3-facility with spacious cages, and were provided with commercial food pellets supplemented with appropriate treats. Drinking water was provided *ad libitum.* Environmental enrichment was provided daily and suffering of the animals was alleviated wherever possible. In 2002, the European Council banned the use of apes for biomedical research in Europe. Yet, this was decided after the initiation of the original vaccine-study described by Rollier *et. al.* [11]. For this reason, the European Council approved continuation of this particular study. The following years, all chimpanzees that were housed at the BPRC were outplaced to public zoos or animal sanctuaries. Before 2001, the use of chimpanzees for biomedical research was highly restricted and regulated, it was not allowed to perform terminal experiments on these animals [12].

Peptide-pools

For stimulation and expansion of HCV-specific cells, two different NS3-peptide-pools were compiled: $NS3_{vaccine}$ was a mixture of 15-mer peptides with 11 amino acids overlap, covering the NS3-sequence of the vaccine constructs; $NS3_{challenge}$ was a mixture of 15-mer peptides with 11 amino acids overlap covering the NS3-variants observed in the inoculum. Identification of NS3 epitopes within NS3 was first performed via the matrix setup [13] and confirmed in a second assay using individual peptides (peptide-sequences are listed in Table S1).

In vitro expansion of NS3-specific T-cell lines

Cells isolated before infection were expanded and tested for response against peptide-pool $NS3_{vaccine}$, whereas cells isolated after challenge were tested against peptide-pool $NS3_{challenge}$. PBMCs isolated at the day of challenge were stimulated in two separate cultures with either $NS3_{vaccine}$ or $NS3_{challenge}$. After thawing, PBMCs were washed and resuspended in culture medium, R20 (RPMI, Invitrogen, CA, USA) supplemented with 20% FCS (MP medicals, Solon, OH, USA), pen/strep (100 U/ml/100 µg/ml) (Invitrogen) and L-glutamine (2mM) (Invitrogen)) and cultured at a final density of 10 million cells per ml in a 24 well culture plate (Greiner Bio-One). The cultures were initiated in R20 containing 5 ng/ml IL-7 (Peprotech) and NS3-peptide-pool (5 µg/ml per peptide). After overnight incubation, 1 ml R20 containing IL-7 (10 ng/ml) and IL-2 (Proleukin Chiron, final concentration 10 IU/ml) was added per well. Cultures were checked daily for general condition and depending on cell density, fresh R20 IL-2/IL-7 was added or cultures were split. After 12 days, the expanded cells were collected and resuspended in fresh R20 for further analysis.

T-cell epitope mapping

The cytokine production profile of HCV-specific T-cell lines was determined by intracellular cytokine staining (ICS) after restimulation with NS3-peptides. For T-cell epitope mapping 0.2 x 10^6 expanded cells were either restimulated with NS3-peptide-pool (1µg/ml/peptide) or without NS3-peptide, in medium containing co-stimulatory aCD28 and aCD49d molecules (2µg each, BD-Biosciences) and 5% FCS (MP-Biomedicals). After 2 hrs, Brefeldin A (Golgiplug 1:1000, BD-Biosciences) was added and 16 hours later the surface markers were stained with a panel of fluorochrome labeled antibodies, containing CD3-PacificBlue (clone SP-34-2, (BD-Pharmingen),

CD14-PE-TexasRed (clone RMO52, BeckmanCoulter), CD20-PE-TexasRed (clone B9E9, BeckmanCoulter), CD4-PE-Cy7 (clone SK3, BD-Pharmingen) and CD8-APC-H7 (clone SK1, BD-Pharmingen). Stained cells were fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences), followed by staining of accumulated intracellular cytokines with IFNγ-APC (clone B27, BD Pharmingen), IL-2-PE (clone MQ1-17H12, BD Pharmingen) and TNFα-FITC (clone MAb11, BD Pharmingen). Cell staining was analyzed on a FACSAria (BD Bioscience) and DIVA software Version 6.1.1.

To evaluate antigen sensitivity, T-cell lines were restimulated with increasing concentrations of individual peptide ranging from 0.05 to 10 µg/ml before intracellular cytokine production was measured.

MHC-class-I genotyping

Animals were genotyped for their MHC-class-I repertoire as previously described [14] (Table 1).

Table 1; MHC class I genotypes of the chimpanzees.

	Patr class I alleles			
	Patr-A		Patr-B	
Vac1	A*03:01	A*04:04	B*02:01	B*03:01
Vac2	A*06:01	A*04:01	B*01:01	B*03:01
Vac3	A*09:01	- [$]	B*01:01	B*02:01
Vac4	A*09:01	A*0101	B*01:01	B*16:0101

[$] Vac3 is likely to be homozygous A*0901

Non-radioactive cytotoxicity assay

NS3-peptide-specific T-cell lines were tested for their capacity to kill peptide-loaded target cells, using a new non-radioactive cytotoxicity assay. In this assay, a panel of matched *Patr* MHC-class-I expressing transfectants, either constructed in 721.221 cells (kindly provided by C. Walker, Center for Vaccines and Immunity, The Ohio State University) or K562 [15] were used as target cells. In brief, 3 x 10^6 MHC-matched transfected target cells were labeled with 1 µM CFSE ($CFSE^{low}$) (Fluka) in polypropylene tubes (Greiner Bio-One) and another 3 x 10^6 MHC-matched transfected target cells,

expressing the same class-I molecule were labeled with 8 µM CFSE ($CFSE^{high}$). Subsequently, $CFSE^{low}$ cells were incubated with medium alone, whereas, $CFSE^{high}$ target cells were pulsed with HCV-peptide (8 µg/ml) for 2 hrs at 37°C. Excess peptide was removed by washing before mixing $CFSE^{high}$ and $CFSE^{low}$ with 1 x 10^6 NS3-specific expanded T-cells (ET ratio 10:1) in a 96 well plate (Greiner Bio-One). The mixed target and effector cells were cocultured for 16 hrs, after which CFSE staining was analyzed using a FACSAria (BD Bioscience) and DIVA software Version 6.1.1. Specific lysis was calculated using the following formula;

% specific lysis = $100-(((high^{peptide}*low^{mock})/(low^{peptide} * high^{mock}))*100)$

and lysis was considered positive when exceeding 25%.

Peptide-binding assay

A cell-based peptide-binding-competition-assay was performed as previously described [15]. In this assay, peptides are tested for binding to transfected cell lines expressing chimpanzee class-I molecules, while competing with a biotin-labeled reference-peptide. In a 96 wells plate, 100.000 MHC class-I transfected K562 or 721.221 cells were incubated with 500nM of the biotinylated-indicator-peptide (in case of A*03:01 ATALECVYK was used, and in case of B*01:01 LSDMHLCSI, in which C indicates the position of the biotin-labeled cysteine) and 0.05 to 100 nM of the peptide of interest, for instance p59 ($NS3_{1258-1272}$), LGFGAYMSK or AATLGFGAY. Peptide KGGLRPRAG, predicted to have no or a very low binding affinity to MHC class I molecules based on the incorporation of glycines on the anchor positions, was used as control. After overnight incubation, excess peptide was removed by washing and indicator-peptide bound to cells was quantified using the DELFIA-system and the Victor3 1420 multilabel counter (Perkin Elmer). The binding affinity of the indicator peptide was verified using its unlabeled peptide as competitor, this is referred to as standardized competitor [15].

RESULTS

Characterization of NS3 specific CD4 and CD8 T-cell cytokine responses

We previously reported [11] an HCV-vaccine evaluation-study in chimpanzees, in which 4 out of 4 immunized animals became viremic shortly after challenge with HCV 1b J4. However, at a later stage, animal Vac1 cleared HCV infection while the three other animals, Vac2, Vac3 and Vac4 became chronically infected. As reported previously, IFNγ release, *ex vivo* lymphoproliferative responses, Th1/Th2 cytokine release patterns, and HCV-specific antibodies were comparable amongst the four animals [11]. NS3 was the immunodominant antigen in all four animals, while the responses against core, E1 and E2 were not only less intense, but also largely absent in Vac1, the animal which was protected against chronic infection. Therefore, functional analysis of the cellular responses was focused on the immunodominant NS3 antigen [11].

After 12 days of culturing in the presence of a pool of NS3 peptides, T-cell lines were restimulated with individual peptides and analyzed for intracellular expression of IFNγ, IL-2 and TNFα using the gating strategy shown in **Figure1**.

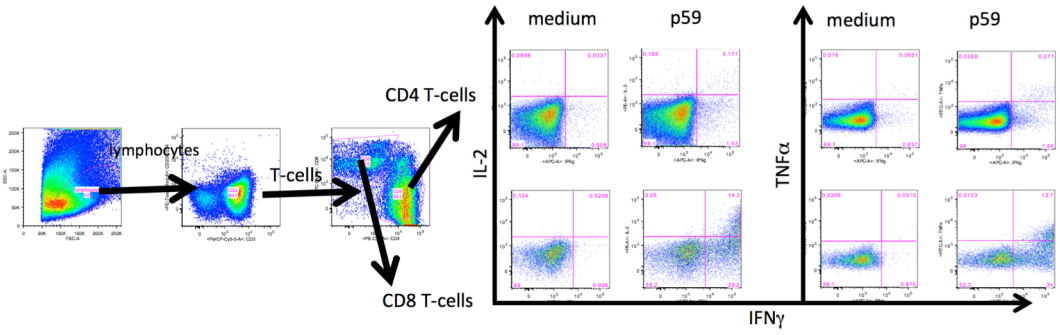


Figure 1; Gating strategy for the evaluation of cytokine production by CD4 and CD8 cells.

Cells from Vac1, two weeks after the last vaccine boost were stimulated with $NS3_{vaccine}$ and restimulated with either $NS3_{1258-1272}$ (p59) or medium alone. Cells within the lymphogate were selected, followed by selection of the CD3 positive, CD20/CD14 negative population, subsequently CD4 and CD8 positive cells were selected. Expression of IFNγ as a function of IL-2 or TNFα was plotted.

Cells within the lymphocyte gate that were CD3 positive, and CD14 and CD20 negative were selected and subsequently IL-2, IFNγ and TNFα single, dual or triple cytokine expression was analyzed in the CD4 and CD8 T-cell subsets within the T-cell lines. Cytokine expression profiles varied per peptide as illustrated in **Figure 1**. For instance, only moderate cytokine induction was observed after peptide specific restimulation of expanded CD4 cells in Vac1. In contract, strong dual IFNγ and IL-2 expression was seen in expanded CD8 T-cells restimulated with peptide 59 ($NS3_{1258-1272}$), while medium alone or peptide 64 (IRTGVRTITTGGPIT) did not induce cytokine production. In comparison, in Vac2 p59 only gave a marginal cytokine induction in expanded CD8 T-cells, while p64 induced moderate dual IFNγ/IL-2 and IFNγ/TNFα cytokine production **(Figure 2)**. Instead, expanded CD4 T-cells in Vac2, showed stronger response towards p59.

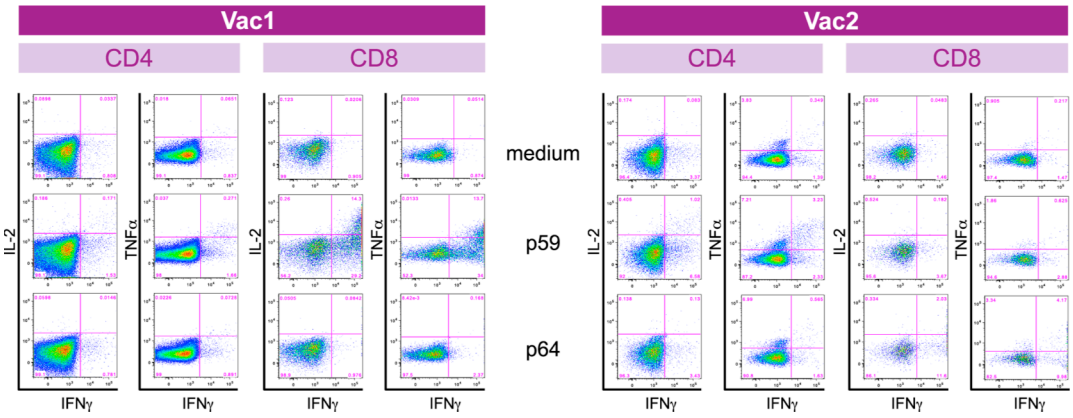


Figure 2; Evaluation of peptide-specific IL-2, IFNγ and TNFα expression in CD4 and CD8 T-cells.

A representative example of the analysis performed in Vac1 (left panel) and Vac2 (right panel), two weeks after the final vaccine boost, is shown. PBMC were first expanded for a 12 day period using a pool of all NS3 peptides, restimulated with individual peptides (medium alone, p59 or p64) and analyzed for induction of IFNγ, IL-2 and TNFα cytokine expression by expanded CD4 and expanded CD8 cells, using the gating strategy described in Figure 1. The numbers in the quadrants, represent the percentage of positive cells calculated from the parent-population.

In this manner the responses against each individual NS3 peptide was investigated and the results are summarized in **Figure 3** in the form of heat maps; showing the percentages of IFNγ producing CD4 **(Figure 3A)** and IFNγ/IL-2 dual cytokine producing CD8 **(Figure 3B)** T-cells in all four animals at all time points tested.

Table 2; Number of NS3- peptides that induce IFNγ production by CD4 T-cells or IL-2/IFNγ dual cytokine production by CD8 T-cells.

		TOTAL	PRE	POST
CD4	**Vac1**	34	34	11
	Vac2	55	42	36
	Vac3	58	51	22
	Vac4	38	19	25
CD8	**Vac1**	22	20	8
	Vac2	22	20	6
	Vac3	35	22	27
	Vac4	44	27	25

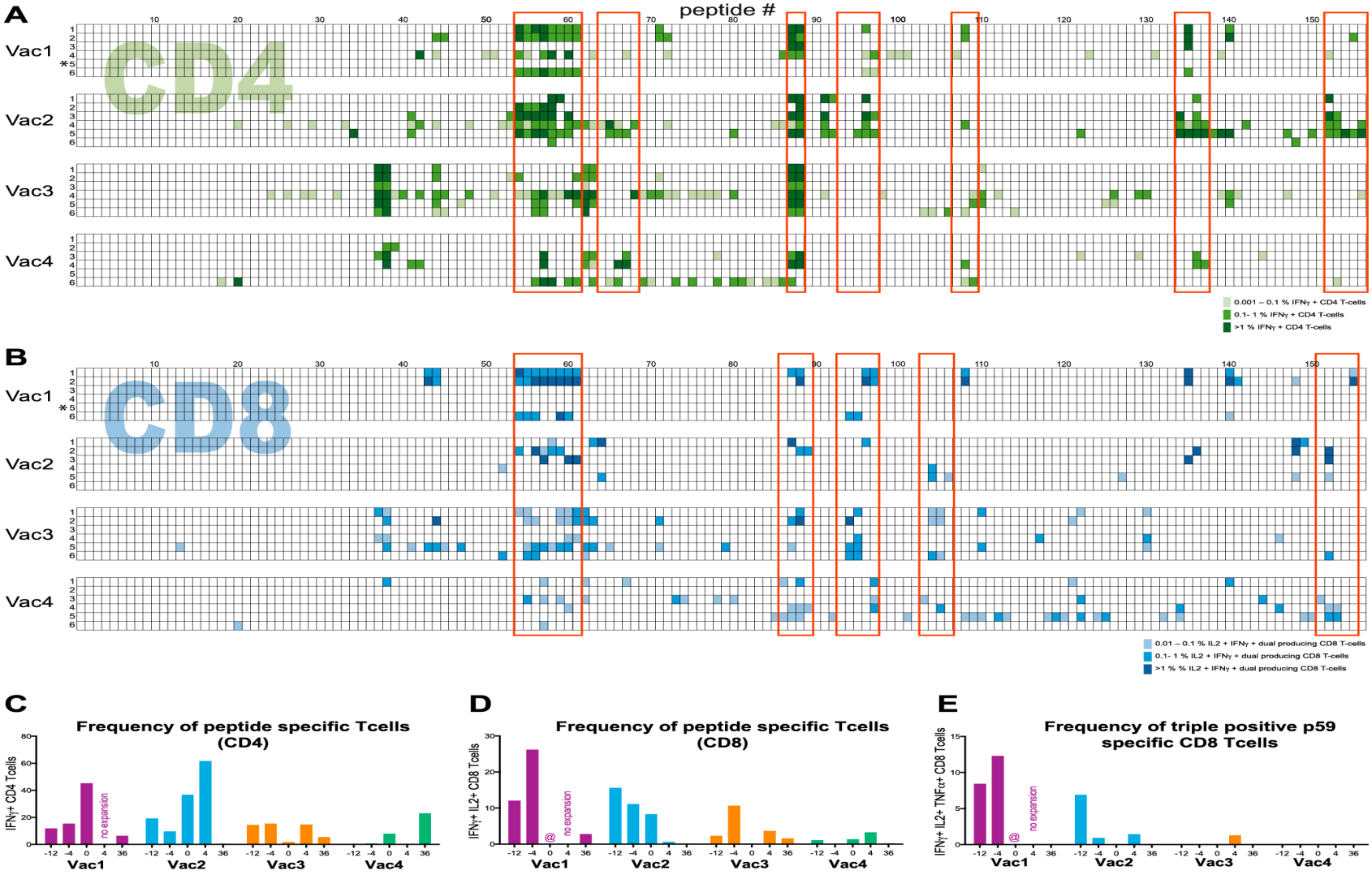
PRE, pre challenge. POST, post challenge

In general, analysis of peptide responses by dual cytokine expression gives a clear-cut distinction between positive and negative responses, because of low background responses. However, for CD4 T-cells the number of dual cytokine expressing cells was often rather low, making it necessary to consider the total number of IFNγ cytokine producing cells. The peptide recognition profiles shown in **Figures 3A and B** indicate that some regions within the NS3 peptide sequence, indicated by red boxes, are broadly recognized amongst the animals. Other peptide specific responses were more restricted and only gave positive responses at some time points in one to two animals. No unique peptide response-pattern could be discerned for Vac1 that could explain vaccine induced viral clearance as compared to Vac2, 3, and 4. Moreover, no relation was observed between the number of peptides recognized and the course of infection (Table 2).

Next, the sum of the % of cytokine producing cells induced by all 156 individual peptides was calculated for expanded CD4 and CD8 lines. This total frequency of peptide-specific IFNγ -producing expanded CD4 T-cells was comparable between Vac1 and Vac2, but somewhat lower in Vac3 and Vac4 **(Figure 3C).** After the last immunization (week -4), total peptide specific CD8 T-cell responses were higher in Vac1 as compared to the other animals **(Figure 3D)**. Triple cytokine production, i.e. simultaneous expression of IFNγ, IL-2 and TNFα by expanded CD8 cells, was also higher in Vac1 and for instance observed in 12.3% of the p59-specific expanded CD8 T-cells 4 weeks prior to HCV infection, whereas this was less than 7% in Vac2, Vac3 and Vac4 **(Figure. 3E)**.

Figure 3; Heat map of NS3-peptide-specific T-cell responses in four immunized chimpanzees at different study time points. (A) Percentage of expanded CD4 T-cells producing I IFNγ upon restimulation with individual peptides, subdivided into three categories; i.e. low response (light green; 0.001%-0.1% specific IFNγ production), intermediate (green; 0.1-1% specific response), high (dark green; > 1% specific response). (B) Percentage of expanded CD8 T-cells showing IL-2/IFNγ double cytokine production upon restimulation with individual peptide, subdivided into three categories; i.e. low response (light blue; 0.01%-0.1% specific IFNγ production), intermediate (blue; 0.1-1% specific response), high (dark blue; > 1% specific response). White areas indicate where no responses were detected. The red boxes highlight areas within NS3 that are broadly recognized. Per animal 6 time points of analysis are shown lined up beneath each other; -12) Two weeks following the 1st MVA boost, cells stimulated with $NS3_{vaccine}$, -4) Four weeks following 2nd MVA boost, cells stimulated with $NS3_{vaccine}$, 0) Day of HCV infection, stimulation with $NS3_{vaccine}$, 0) Day of HCV infection, stimulation with $NS3_{challenge}$, and 4 and 36 weeks after challenge, stimulation with $NS3_{challenge}$. The individual peptides tested are indicated at the top of the map by the numbers 1 to 156. The red boxes represent broadly recognized regions within NS3.
Total frequency (all peptide responses combined) of (C) IFNγ production by expanded CD4 T-cells and (D) IFNγ/IL2 dual cytokine production by expanded CD8 T-cells per animal. (E) Frequency of p59 specific triple positive IL2/TNFα/IFNγ producing CD8 T-cells. The numbers on the X-axis represent the study week, relative to time of challenge.
*Despite several attempts, frozen cells from Vac1 from 4 weeks following HCV infection did not respond to either $NS3_{vaccine}$ or $NS3_{challenge}$ peptide stimulation and no expansion could be achieved $^{@}$Due to a high IFNγ background. specifiv responses could not be detected.

Figure 2


A
peptide #
Vac1
Vac2
Vac3
Vac4
CD4
0.001 – 0.1 % IFNγ + CD4 T-cells
0.1- 1 % IFNγ + CD4 T-cells
>1 % IFNγ + CD4 T-cells
B
CD8
0.01 – 0.1 % IL2 + IFNγ + dual producing CD8 T-cells
0.1- 1 % IL2 + IFNγ + dual producing CD8 T-cells
>1 % IL2 + IFNγ + dual producing CD8 T-cells
C
Frequency of peptide specific Tcells (CD4)
IFNγ+ CD4 Tcells
no expansion
D
Frequency of peptide specific Tcells (CD8)
IFNγ+ IL2+ CD8 Tcells
E
Frequency of triple positive p59 specific CD8 Tcells
IFNγ+ IL2+ TNFα+ CD8 Tcells

In conclusion, we observed increased number of NS3 specific cytokine producing expanded CD8 T-cells after the last immunization, and especially more triple IFNγ, IL-2 and TNFα cytokine producing cells in the animal that cleared the virus, relative to those animals that became chronically infected**.**

Cytolytic capacity of NS3 p59 specific CD8 T-cells in Vac1

Next, NS3-peptide-specific cells were evaluated for their capacity to kill peptide-loaded target cells. Similar to standard ^{51}chromium release assays, T-cells were expanded prior to analysis [6, 16]. The expanded T-cells were tested for their lytic capacity for all individual peptides that were found to induce IL-2/IFNγ dual expression in expanded CD8 cells **(Figure 2B)** and was tested against a panel of MHC matched target cells [15] (Table 1).

Figure 4A illustrates specific killing of p59-pulsed CFSE^{high} relative to unpulsed CFSE^{low} Patr-A*03:01 target cells, using expanded T-cells from Vac1, isolated 10 weeks after HCV challenge. As a control, no difference in ratio of the area under the curve was observed if both the CFSE^{high} and CFSE ^{low} Patr-A*03:01 targets were unpulsed **(Figure 4C)**, precluding non-specific toxicity due to labeling with higher CFSE concentration. Killing of target cells was only observed for particular peptide-MHC class I combinations. As shown in **Figures 4B and D**, neither unpulsed nor p59-pulsed Patr-B*02:01 targets were lysed by expanded cells from Vac1. Amongst all peptides tested, i.e. 24, 22 and 35 in Vac1, Vac2 and Vac3 respectively, highly efficient killing was only observed against p59 in Vac1 and only when this peptide was presented by Patr-A*03:01 **(Figure 4E to G).** Only the peptides partly overlapping with p59 gave some lysis, while none of the other MHC class-I-peptide-combinations exceeded 25% of specific killing. Due to the limited number of cells after expansion, Vac4 could not be tested.

Collectively, the data show that although stimulation with p59 can induce some level of cytokine production in all vaccinees, a p59-mediated cytolytic capacity was only observed in Vac1.

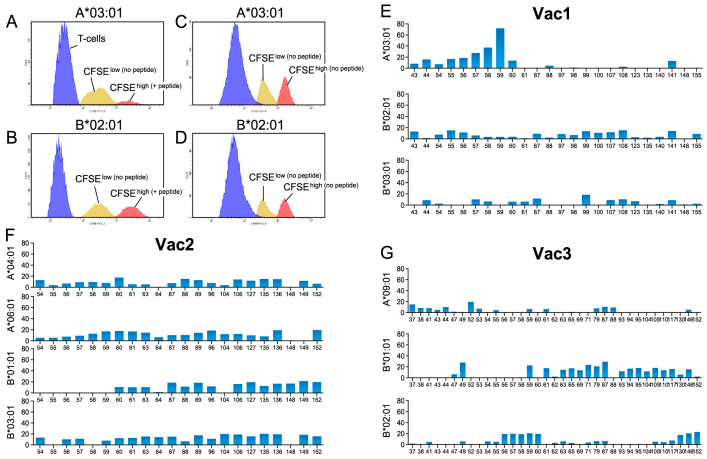


Figure 4; Cytolytic killing of CFSE-loaded target cells.
(A) Specific lysis of $NS3_{1258-1272}$-pulsed Patr-A*03:01 target cells (red peak) in comparison with unpulsed Patr-A*03:01 target cells (yellow peak) by NS3-peptide-pool expanded PBMC from Vac1 isolated 10 weeks after HCV 1bJ4 challenge (blue peak). (B) No specific lysis of $NS3_{1258-1272}$-pulsed Patr-B*02:01 target cells. (C) and (D) Control experiments showing no killing of unpulsed Patr-A*03:01 or Patr-B*02:01 target cells. (E,F,G) Percentage of lysis for all MHC class-I-peptide-combinations tested in Vac1, Vac2, Vac3 respectively.

NS3p59$_{(aa1258-1272)}$ binds with high affinity to Patr-A*03:01.

Peptide binding to MHC-class I is determined by the anchor-residues fitting into the B- and the F-pocket of the relevant allotype for most MHC class-I-molecules. Usually MHC class I molecules bind nonamer peptides, in which the p2-position of the peptide anchors into the B-pocket and the p9-position of the peptide anchors into the F-pocket. Peptide 59, which was observed to initiate cytolysis when presented in the context of Patr-A*03:01, was scanned using the NetMHCpan version 2.8 prediction algorithm [17] against the MHC class I molecules present in the studied cohort (Table 1). It is known that peptides that bind, or are predicted to bind to MHC class I molecules with an affinity < 500 nm are potential T-cell epitopes [18-20]. In p59 four potential nonamer peptides are present, AATLGFGAY, ATLGFGAYM, TLGFGAYMS, and LGFGAYMSK. The only combination that was predicted to bind with an affinity < 500 nm was LGFGAYMSK in the context of Patr-A*03:01. A cell-based peptide binding competition assay was performed to test the binding-affinity of p59, and the nonamer peptides AATLGFGAY and LGFGAYMSK to Patr-A*03:01 and as control to Patr-B*01:01. The peptide binding motif of Patr-A*03:01 is defined as X(STA)XXXXXX(RK) and for Patr-B*01:01 as (X(ST)XXXXXX(IL) [15, 21]. **Figure 5** shows that all three NS3 peptides have a high binding affinity to Patr-A*03:01, with IC_{50} values between 0.03 to 0.2 µM, which is higher compared to the control peptide (IC_{50}=8.2 µM) and the standardized competitor peptide of Patr-A*03:01 with IC_{50}=1.5 µM [15].

Peptide LGFGAYMSK was found to have the highest binding affinity to Patr-A*03:01. In contrast, only low binding affinity was observed for p59 in the context of Patr-B*01:01 as compared to its standardized competitor (IC_{50}=1.0 µM), whereas for the two nonamer peptides, no amenable regression curves could be drawn suggesting low or no binding affinity (not shown).

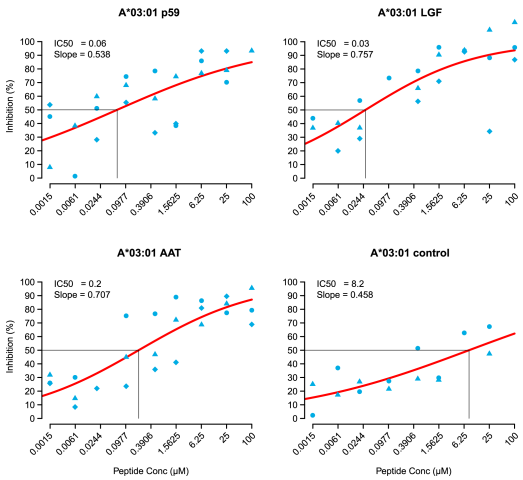


Figure 5; Dose-inhibition curves for selected NS3-peptides to Patr-A*03:01
Binding affinity of selected NS3-peptides to Patr-A*03:01, established by peptide binding competition assay. Patr-A*0301 cells were incubated with increasing concentrations of NS3 p59 (top left), LGFGAYMSK (LGF, top right), AATLGFGAY (AAT, bottom left), or KGGLRPRAG (control, bottom right) in the presence of biotin-labeled indicator peptide.
Binding of indicator peptide was quantified by incubation with europium labeled streptavidin. Indicated is reduction in percentage of europium positive cells relative to cells incubated with indicator-peptide only. The indicated IC_{50} values (μM) are derived from the regression curves of three independent experiments in the case of p59, LGF and AAT, and of two individual experiments in the case of KGG. The IC_{50} values were estimated using non-linear least-squares regression with the "R" platform for statistical computing.

T-cells of Vac1 respond at lower peptide concentration

Antigen specific responses not only depend on the presentation of peptides by MHC class I molecules but also on the binding of the peptide/MHC complex to the T-cell-receptor (TCR). Therefore, we tested whether the response-threshold of CD4 and CD8 T-cell lines from Vac1 differed from Vac2 and Vac3. To this end, NS3-specific expanded T-cells, isolated 4 weeks prior to HCV infection, were expanded from PBMCs, restimulated with different concentrations of $NS3_{1258-1272}$ ranging from 0.05 to 10 µg/ml, and stained for intracellular IL-2 and IFNγ.. Expanded CD8 T-cells from Vac1 were found to respond at a peptide concentration as low as 0.05 µg/ml whereas, at least, 20 times more peptide was required for CD8 T-cells from Vac2 and Vac3 **(Figure 6)**. For IFNγ production by expanded CD4 T-cells the difference is even more striking. Here, we could hardly detect p59-specific IFNγ producing CD4 T-cells in Vac2 and Vac3 while these cells are abundantly present in Vac1.

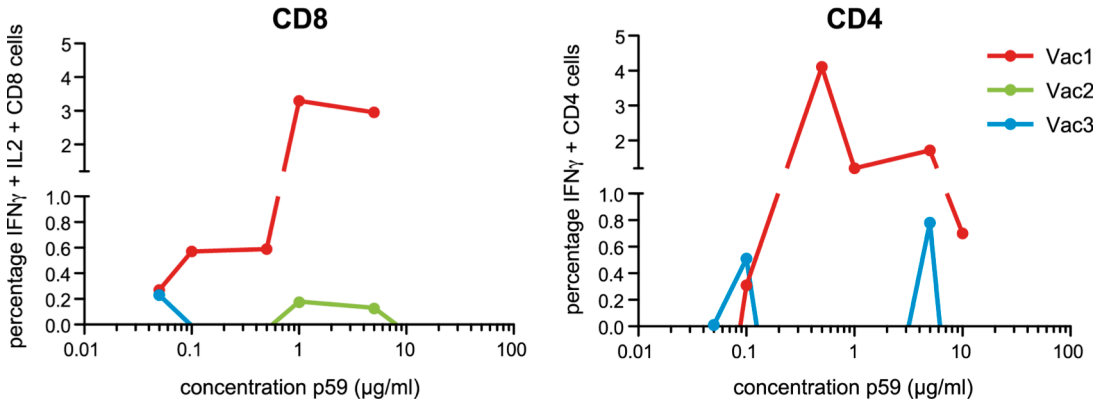


Figure 6; Antigen sensitivity of p59 specific cytokine induction.
NS3-expanded T-cells from Vac1, Vac2 and Vac3, from 4 weeks prior to HCV challenge, were restimulated with a concentration range of p59 and evaluated for induction of IL-2/ IFNγ expressing CD8 T-cells (left graph) and IFNγ expressing CD4 T-cells (right graph).

DISCUSSION

The chimpanzee is the closest living relative of man and the only validated animal model for HCV infection. The evolutionary proximity of both species is reflected by close genetic and immunological similarity. For these reasons the chimpanzee provides an exquisite animal model, in which immune responses against HCV vaccines and the effect on deliberate infection can be investigated in an experimental setting [2]. Here, we report of an in depth profiling of CD4 and CD8 T-cell responses in four chimpanzees, which were immunized with an experimental vaccine for HCV and subsequently challenged with HCV 1b J4 [11].

While all four animals became infected, only the Patr-A*03:01 positive animal was able to clear HCV infection. Detailed mapping of CD4 and CD8 T-cell cytokine responses, and the analysis of their cytolytic potential, revealed that expanded T-cells of the vaccinee which effectively cleared HCV infection displayed: a) a higher frequency of peptide-specific IL-2/IFNγ dual cytokine producing CD8 T-cells; b) peptide-specific TNFα production in CD8 T-cells; c) cytolytic capacity against peptide-loaded target cells; d) cytokine induction at a low antigen concentration for the cytolysis-inducing peptide. Remarkably, the cytolysis-inducing peptide p59 was found to bind strongly to Patr-A*03:01, which was only present in Vac1. Using the NetMHCpan algorithm, it was predicted that peptide LGFGAYMSK, one of the nonamer peptides present in the 15-mer p59, would show the strongest binding to Patr-A*03:01. Peptide LGFGAYMSK was also predicted to bind to the MHC class I molecules Patr-A*01:01, A*04:01, A*04:04, and A*09:01, present in all of the studied animals, but only with intermediate affinity between 0.9 and 5 μM. These data are in agreement with the differences in cytokine responses and cytolyses reported in expanded T-cells here and could indicate a particular affinity threshold is necessary for a peptide to induce adequate immune responses for combating HCV. The strong binding of the 15-mer p59 to Patr-A*03:01 is somewhat unexpected, as generally nonamer peptides bind to MHC class I molecules. Nonetheless, in humans it has been shown that particular MHC class I molecules are capable of binding longer peptides with bulged structures [22, 23]. Stimulation assays would give information about the biological relevance of the binding experiments. However, the limited number of cells stored from these animals, preclude performing such assays.

Unlike *ex vivo* detection of CTL in humans, detection of these cells in chimpanzees can often only be achieved after amplification of antigen specific responses [6, 16]. Obviously, *in vitro* expansion of T-cells from frozen PBMC has its limitations. Antigen presenting cells may be affected by the freezing/thawing process resulting in –partly hampered- antigen presentation. Furthermore, the addition of cytokines and peptides may skew the immune system differently as compared to the *in vivo* situation.

To determine the degranulating capacity of T-cells, CD107a stainings were performed on T-cell lines. However, the results of these assays were unclear and no conclusion could be drawn from it, basically because of the lack of a proper negative control.

Unlike in the intracellular cytokine staining assays, where the difference between cells restimulated with peptide or medium alone was very clear, no clear difference could be observed when CD107a was used. Probably the expansion-protocol used here does agree with antigen specific CD107a staining. Unfortunately, the number of stored cells from these animals is limited and assays like this require too many cells to repeat the assays.
And although we cannot exclude that upon further amplification of antigen specific responses, CTL responses might also be detectable in the other animals, our data suggest that they are of lower magnitude.

To study the possibility of viral escape from immune pressure as a cause of development of chronicity in Vac2, Vac3 and Vac4, analysis of viral sequences was performed and compared with the inoculum sequence (data not shown). Only a limited number of amino acid substitutions were detected in the areas that overlap with cytokine-inducing peptides. There was however no evidence for mutational escape in the immunodominant p59 in any of the animals.

This study highlights a potential role for CD8 mediated cytolytic responses as a discriminatory factor between viral clearance and the development of chronic infection in HCV vaccinated subjects and underscores that this parameter is to be considered as an important immunological readout in future vaccine trials. Indeed, studying peptide binding and CD8 cytolytic responses could complement the other contributing immune mechanisms previously described [11, 24, 25].

It should be noted that the reported data were obtained from peripheral blood and not from the site of HCV-induced inflammation, the liver. For ethical reasons and animal welfare, the number of liver biopsies taken during the study, was limited and not sufficient for the comprehensive analysis as presented in this manuscript. Nevertheless, a direct comparison of CD8 T-cell function between liver and peripheral blood in chimpanzees during HCV infection, showed in essence that the cells in the circulation reflect the situation in the liver [26, 27].

The data presented here, are in line with the finding that self-limiting HCV infection in humans is associated with the presence of HLA-B*57 and HLA-B*27 restricted CD8 T-cell responses [28, 29]. Chimpanzee Patr-A*03:01 and Patr-B*01:01 have almost identical B-pocket residues as compared to HLA-B*57. Moreover, peptide binding studies have shown that both Patr-molecules may potentially bind HLA-B*57 or HLA-B*27 [15]

In conclusion, we observed that in contrast to peptide-specific cytokine production, the induction of an effective CD8 T-cell effector function elicited in the context of peptide presentation by appropriate class-I molecules appeared to be associated with clearance of infection.

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pplementary Table S1
ptide numbering of Vaccine- and Challenge-sequence relative to HCV reference strain H77

H77	p#	$NS3_{vaccine}$-sequence	$NS3_{challenge}$-sequence	H77	p#	$NS3_{vaccine}$-sequence	$NS3_{challenge}$-sequence
1026	1	MAPITAYSQQTRGLL	MAPITAYSQQTRGLL	1338	79	TAGARLVVLATATPP	TAGARLVVLATATPP
1030	2	TAYSQQTRGLLGCII	TAYSQQTRGLLGCII	1342	80	RLVVLATATPPGSIT	RLVVLATATPPGSIT
1034	3	QQTRGLLGCIITSLT	QQTRGLLGCIITSLT	1346	81	LATATPPGSITVPHP	LATATPPGSITVPHP
1038	4	GLLGCIITSLTGRDK	GLLGCIITSLTGRDK	1350	82	TPPGSITVPHPNIEE	TPPGSITVPHPNIEE
1042	5	CIITSLTGRDKNQVD	CIITSLTGRDKNQVD	1354	83	SITVPHPNIEEVALS	SITVPHPNIEE**I**ALS
1046	6	SLTGRDKNQVDGEVQ	SLTGRDKNQVDGEVQ	1358	84	PHPNIEEVALSNTGE	PHPNIEE**I**ALSNTGE
1050	7	RDKNQVDGEVQVLST	RDKNQVDGEVQVLST	1362	85	IEEVALSNTGEIPFY	IEE**I**ALSNTGEIPFY
1054	8	QVDGEVQVLSTATQS	QVDGEVQVLSTAT**H**S	1366	86	ALSNTGEIPFYGKAI	ALSNTGEIPFYGKAI
1058	9	EVQVLSTATQSFLAT	EVQVLSTAT**H**SFLAT	1370	87	TGEIPFYGKAIPIEA	TGEIPFYGKAIPIEA
1062	10	LSTATQSFLATCVNG	LSTAT**H**SFLATCVNG	1374	88	PFYGKAIPIEAIKGG	PFYGKAIPIEAIKGG
1066	11	TQSFLATCVNGVCWT	T**H**SFLATCVNGVCWT	1378	89	KAIPIEAIKGGRHLI	KAIPIEAIKGGRHLI
1070	12	LATCVNGVCWTVYHG	LATCVNGVCWTVYHG	1382	90	IEAIKGGRHLIFCHS	IEAIKGGRHLIFCHS
1074	13	VNGVCWTVYHGAGSK	VNGVCWTVYHGAGSK	1386	91	KGGRHLIFCHSKKKC	KGGRHLIFCHSKKKC
1078	14	CWTVYHGAGSKTLAG	CWTVYHGAGSKTLAG	1390	92	HLIFCHSKKKCDELA	HLIFCHSKKKCDELA
1082	15	YHGAGSKTLAGPKGP	YHGAGSKTLAGPKGP	1394	93	CHSKKKCDELAAKLT	CHSKKKCDELAAKLT
1086	16	GSKTLAGPKGPITQM	GSKTLAGPKGPITQM	1398	94	KKCDELAAKLTGLGL	KKCDELAAKLTGLGL
1090	17	LAGPKGPITQMYTNV	LAGPKGPITQMYTNV	1402	95	ELAAKLTGLGLNAVA	ELAAKLTGLGLNAVA
1094	18	KGPITQMYTNVDQDL	KGPITQMYTNVD**L**DL	1406	96	KLTGLGLNAVAYYRG	KLTGLGLNAVAYYRG
1098	19	TQMYTNVDQDLVGWP	TQMYTNVD**L**DLVGWP	1410	97	LGLNAVAYYRGLDVS	LGLNAVAYYRGLDVS
1102	20	TNVDQDLVGWPAPPG	TNVD**L**DLVGWPAPPG	1414	98	AVAYYRGLDVSVIPT	AVAYYRGLDVSVIP**P**
1106	21	QDLVGWPAPPGARSM	**L**DLVGWPAPPGARSM	1418	99	YRGLDVSVIPTSGDV	YRGLDVSVIP**P**SGDV
1110	22	GWPAPPGARSMTPCT	GWPAPPGARSMTPCT	1422	100	DVSVIPTSGDVVVVA	DVSVIP**P**SGDVVVVA
1114	23	PPGARSMTPCTCGSS	PPGARSMTPCTCGSS	1426	101	IPTSGDVVVVATDAL	IP**P**SGDVVVVATDAL
1118	24	RSMTPCTCGSSDLYL	RSMTPCTCGSSDLYL	1430	102	GDVVVVATDALMTGF	GDVVVVATDALMTGF
1122	25	PCTCGSSDLYLVTRH	PCTCGSSDLYLVTRH	1434	103	VVATDALMTGFTGDF	VVATDALMTGFTGDF
1126	26	GSSDLYLVTRHADVI	GSSDLYLVTRHADVI	1438	104	DALMTGFTGDFDSVI	DALMTGFTGDFDSVI
1130	27	LYLVTRHADVIPVRR	LYLVTRHADVIPVRR	1442	105	TGFTGDFDSVIDCNT	TGFTGDFDSVIDCNT
1134	28	TRHADVIPVRRRGDS	TRHADVIPVRRRGDS	1446	106	GDFDSVIDCNTCVTQ	GDFDSVIDCNTCVTQ
1138	29	DVIPVRRRGDSRGSL	DVIPVRRRGDSRGSL	1450	107	SVIDCNTCVTQTVDF	SVIDCNTCVTQTVDF
1142	30	VRRRGDSRGSLLSPR	VRRRGDSRGSLLSPR	1454	108	CNTCVTQTVDFSLDP	CNTCVTQTVDFSLDP
1146	31	GDSRGSLLSPRPVSY	GDSRGSLLSPRPVSY	1458	109	VTQTVDFSLDPTFTI	VTQTVDFSLDPTFTI
1150	32	GSLLSPRPVSYLKGS	GSLLSPRPVSYLKGS	1462	110	VDFSLDPTFTIETTT	VDFSLDPTFTIETTT
1154	33	SPRPVSYLKGSSGGP	SPRPVSYLKGSSGGP	1466	111	LDPTFTIETTTVPQD	LDPTFTIETTTVPQD
1158	34	VSYLKGSSGGPLLCP	VSYLKGSSGGPLLCP	1470	112	FTIETTTVPQDAVSR	FTIETTTVPQDAVSR
1162	35	KGSSGGPLLCPSGHV	KGSSGGPLLCPSGHV	1474	113	TTTVPQDAVSRSQRR	TTTVPQDAVSRSQRR
1166	36	GGPLLCPSGHVVGIF	GGPLLCPSGHVVGIF	1478	114	PQDAVSRSQRRGRTG	PQDAVSRSQRRGRTG
1170	37	LCPSGHVVGIFRAAV	LCPSGHVVGIFRAAV	1482	115	VSRSQRRGRTGRGRS	VSRSQRRGRTGRGRS

Supplementary Table S1 (continue)
Peptide numbering of Vaccine- and Challenge-sequence relative to HCV reference strain H77

H77	p#	$NS3_{vaccine}$-sequence	$NS3_{challenge}$-sequence	H77	p#	$NS3_{vaccine}$-sequence	$NS3_{challenge}$-sequence
1186	41	TRGVAKAVDFIPVES	TRGVAKAVDFIPVES	1498	119	IYRFVTPGERPSGMF	IYRFVTPGERPSGMF
1190	42	AKAVDFIPVESMETT	AKAVDFIPVESMETT	1502	120	VTPGERPSGMFDSSV	VTPGERPSGMFDSSV
1194	43	DFIPVESMETTMRSP	DFIPVESMETTMRSP	1506	121	ERPSGMFDSSVLCEC	ERPSGMFDSSVLCEC
1198	44	VESMETTMRSPVFTD	VESMETTMRSPVFTD	1510	122	GMFDSSVLCECYDAG	GMFDSSVLCECYDAG
1202	45	ETTMRSPVFTDNSSP	ETTMRSPVFTDNSSP	1514	123	SSVLCECYDAGCAWY	SSVLCECYDAGCAWY
1206	46	RSPVFTDNSSPPAVP	RSPVFTDNSSPPAVP	1518	124	CECYDAGCAWYELTP	CECYDAGCAWYELTP
1210	47	FTDNSSPPAVPQTFQ	FTDNSSPPAVPQTFQ	1522	125	DAGCAWYELTPAETT	DAGCAWYELTPAETT
1214	48	SSPPAVPQTFQVAHL	SSPPAVPQTFQVAHL	1526	126	AWYELTPAETTVRLR	AWYELTPAETTVRLR
1218	49	AVPQTFQVAHLHAPT	AVPQTFQVAHLHAPT	1530	127	LTPAETTVRLRAYLN	LTPAETTVRLRAYLN
1222	50	TFQVAHLHAPTGSGK	TFQVAHLHAPTGSGK	1534	128	ETTVRLRAYLNTPGL	ETTVRLRAYLNTPGL
1226	51	AHLHAPTGSGKSTKV	AHLHAPTGSGKSTKV	1538	129	RLRAYLNTPGLPVCQ	RLRAYLNTPGLPVCQ
1230	52	APTGSGKSTKVPAAY	APTGSGKSTKVPAAY	1542	130	YLNTPGLPVCQDHLE	YLNTPGLPVCQDHLE
1234	53	SGKSTKVPAAYAAQG	SGKSTKVPAAYAAQG	1546	131	PGLPVCQDHLEFWES	PGLPVCQDHLEFWES
1238	54	TKVPAAYAAQGYKVL	TKVPAAYAAQGYKVL	1550	132	VCQDHLEFWESVFTG	VCQDHLEFWESVFTG
1242	55	AAYAAQGYKVLVLNP	AAYAAQGYKVLVLNP	1554	133	HLEFWESVFTGLTHI	HLEFWESVFTGLTHI
1246	56	AQGYKVLVLNPSVAA	AQGYKVLVLNPSVAA	1558	134	WESVFTGLTHIDAHF	WESVFTGLTHIDAHF
1250	57	KVLVLNPSVAATLGF	KVLVLNPSVAATLGF	1562	135	FTGLTHIDAHFLSQT	FTGLTHIDAHFLSQT
1254	58	LNPSVAATLGFGAYM	LNPSVAATLGFGAYM	1566	136	THIDAHFLSQTKQAG	THIDAHFLSQTKQAG
1258	59	VAATLGFGAYMSKAH	VAATLGFGAYMSKAH	1570	137	AHFLSQTKQAGDNFP	AHFLSQTKQAGDNFP
1262	60	LGFGAYMSKAHGIEP	LGFGAYMSKAHGIEP	1574	138	SQTKQAGDNFPYLVA	SQTKQAGDNFPYLVA
1266	61	AYMSKAHGIEPNIRT	AYMSKAHGIEPNIRT	1578	139	QAGDNFPYLVAYQAT	QAGDNFPYLVAYQAT
1270	62	KAHGIEPNIRTGVRT	KAHGIEPNIRTGVRT	1582	140	NFPYLVAYQATVCAR	NFPYLVAYQATVCAR
1274	63	IEPNIRTGVRTITTG	IEPNIRTGVRTITTG	1586	141	LVAYQATVCARAQAP	LVAYQATVCARAQAP
1278	64	IRTGVRTITTGGPIT	IRTGVRTITTGG**S**IT	1590	142	QATVCARAQAPPPSW	QATVCARAQAPPPSW
1282	65	VRTITTGGPITYSTY	VRTITTGG**S**ITYSTY	1594	143	CARAQAPPPSWDQMW	CARAQAPPPSWDQMW
1286	66	TTGGPITYSTYGKFL	TTGG**S**ITYSTYGKFL	1598	144	QAPPPSWDQMWKCLI	QAPPPSWDQMWKCLI
1290	67	PITYSTYGKFLADGG	**S**ITYSTYGKFLADGG	1602	145	PSWDQMWKCLIRLKP	PSWDQMWKCLIRLKP
1294	68	STYGKFLADGGCSGG	STYGKFLADGGCSGG	1606	146	QMWKCLIRLKPTLHG	QMWKCLIRLKPTLHG
1298	69	KFLADGGCSGGAYDI	KFLADGGCSGGAYDI	1610	147	CLIRLKPTLHGPTPL	CLIRLKPTLHGPTPL
1302	70	DGGCSGGAYDIIICD	DGGCSGGAYDIIICD	1614	148	LKPTLHGPTPLLYRL	LKPTLHGPTPLLYRL
1306	71	SGGAYDIIICDECHS	SGGAYDIIICDECHS	1618	149	LHGPTPLLYRLGAVQ	LHGPTPLLYRLGAVQ
1310	72	YDIIICDECHSTDWT	YDIIICDECHSTD**S**T	1622	150	TPLLYRLGAVQNEIT	TPLLYRLGAVQNEI**I**
1314	73	ICDECHSTDWTTILG	ICDECHSTD**S**TTILG	1626	151	YRLGAVQNEITLTHP	YRLGAVQNEI**I**LTHP
1318	74	CHSTDWTTILGIGTV	CHSTD**S**TTILGIGTV	1630	152	AVQNEITLTHPITKF	AVQNEI**I**LTHPITKF
1322	75	DWTTILGIGTVLDQA	D**S**TTILGIGTVLDQA	1634	153	EITLTHPITKFVMAC	EI**I**LTHPITKFVMAC
1326	76	ILGIGTVLDQAETAG	ILGIGTVLDQAETAG	1638	154	THPITKFVMACMSAD	THPITKFVMACMSAD
1330	77	GTVLDQAETAGARLV	GTVLDQAETAGARLV	1642	155	TKFVMACMSADLEVV	TKFVMACMSADLEVV
1334	78	DQAETAGARLVVLAT	DQAETAGARLVVLAT	1646	156	MACMSADLEVVT	MACMSADLEVVT

In red are indicated the amino acids in the NS3challenge sequence that are different from the NS3vaccine sequence

2.3

Clearance Of Genotype 1b Hepatitis C Virus In Chimpanzees In The Presence Of Vaccine-induced E1 Neutralizing Antibodies.

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ABSTRACT.

Accumulating evidence indicates that neutralizing antibodies play an important role in protection from chronic Hepatitis C Virus (HCV) infection. Efforts to elicit such responses by immunization with intact heterodimeric E1E2 envelope proteins have met with limited success. To determine if antigenic sites, which are not exposed by the combined E1E2 heterodimer structure, are capable of eliciting neutralizing antibody responses, we expressed and purified each as separate recombinant proteins E1, and E2 from which the immunodominant hypervariable region (HVR-1) was deleted. Immunization of chimpanzees with either E1 or E2 alone induced antigen specific T-helper cytokines of similar magnitude. Unexpectedly the capacity to neutralize HCV was observed in E1 but not in animals immunized with E2 devoid of HVR-1. Furthermore, *in vivo* only E1 vaccinated animals exposed to the heterologous HCV-1b inoculum cleared HCV infection.

INTRODUCTION

The Hepatitis C Virus (HCV) envelope glycoproteins, E1 and E2, are heterodimerically exposed on the surface of the virion and play a crucial role in receptor binding, viral fusion and entry of HCV [1]. Combined, E1E2 heterodimers have long been considered and evaluated as candidate HCV vaccine antigens, primarily, but not exclusively for the induction of neutralizing antibodies [2]. Due to the different functions of the E1E2 heterodimer it is safe to assume that it will undergo conformational changes during the virus life cycle [3]. The E2 molecule in this complex is known to bind specifically to cell-surface molecules such as CD81, SR-B1, Claudin-1 and others [1, 4]. However, both in E1 and E2 structural homologies to fusion mediating proteins of related viral families have been described [5]. E2 also possesses a major determinant of isolate specific neutralizing antibodies located near its N-terminus called the hypervariable region (HVR-1). However, due to its immunodominance and the consequential selective pressure on this region, it rapidly accumulates non-synonymous mutations making it hypervariable which is an undesirable attribute for a candidate vaccine antigen. In contrast, the role of E1 in HCV infection and immunity is still unclear, yet several antibodies directed against E1 were found to prevent cell entry [6, 7].

We rationalized that by removing the HVR-1 from E2, and separating the two components of the heterodimer, that novel structural features might be revealed, creating new targets for the induction of broad neutralizing antibodies. To test this hypothesis, chimpanzees were immunized with either recombinant E2 protein with the HVR-1 deleted, or the intact recombinant E1 protein alone. To determine if the vaccine induced antibody responses were sufficient to protect from persistent HCV infection, all animals were exposed to a 1b inoculum, which has the propensity to cause chronic infection. By 18 weeks the two E1 immunized animals had cleared HCV infection, while RNA viremia persisted in the two E2 immunized animals and the control animal. Vaccine induced protection from persistent HCV infection correlated with E1 induced neutralizing antibodies, demonstrating a previously unrecognized role for E1 subunit in immunization.

MATERIALS & METHODS

Animals

This study was critically reviewed and approved and undertaken by the institute's animal ethical committee and performed in accordance with Dutch law and international guidelines for the use of animals in research (BPRC IACUC ID #253) in consultation and prior to amended Dutch legislation. Five mature, captive bred chimpanzees (Pan troglodytes verus), naive for retroviruses and hepatitis viruses, were socially housed and monitored multiple times per day by experienced veterinary staff (BPRC, Rijswijk, The Netherlands).

Throughout the study, animals were checked twice daily for general behavior, appetite and stool consistency. Body weight, rectal temperature and hematology and clinical chemistry were assessed during each sedation for immunization, blood collection or liver biopsy. Serum and peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples collected using aseptic techniques as previously described [8].

HCV immunogens and assay antigens

Recombinant HCV envelope proteins E1s (aa192 to aa326) and E2ΔHVR-1 (E2 aa412 to aa715 with deletion of HVR-1 aa384 to aa411) were used for immunizations and in the cellular assays. Both proteins were derived from the European HCV 1b BE11 strain and were expressed by recombinant vaccinia virus transfected Vero cells, purity of all proteins was estimated to be >95% by SDS-PAGE [9]. For immunization, E1 and E2 proteins were formulated with Alum (Alhydrogel, Superfos Biosector, Denmark), 1.3% $Al(OH)_3$ in buffered phosphate solution and diluted with physiological saline to a final protein concentration of 50 µg/ml and 0.13% $Al(OH)_3$. Although both the vaccine proteins as well as the challenge virus were derived from genotype HCV 1b, the challenge was considered heterologous since in E1 7%, and in E2 approximately 11% of the amino acid residues were different between the vaccine and challenge sequence.

To analyze virus-induced immune responses after challenge, HCV NS3 (NS3 1071 to 1084 and 1181 to 1465), an E. coli fusion protein based on a HCV 1b isolate (BE8309), was used.

Experimental Design, immunizations and HCV exposure

Chimpanzee E1-Ma and E1-Yo were immunized with 50µg E1, while E2-Jo and E2-Ka received 50µg E2 recombinant protein. One animal, Ctrl-Hu, served as a challenge control and did not receive any HCV immunogen or adjuvant before challenge.

Animals were given intramuscular (I.M.) immunizations in the biceps at weeks 0, 3, 6, 9, 12 and 15 at a dose of 50µg protein per ml diluted Alum **(Figure 1)**. At week 18, the animals were intravenously (I.V.) exposed to 100 CID of an *in vitro* titrated HCV 1b inoculum J4.91'01, diluted in saline.

Humoral responses

Vaccine specific antibody titers were determined by limiting dilution ELISA [8] or INNO-LIA HCV (Innogenetics, Ghent , Belgium).

HCV neutralizing capacity of longitudinal serum samples was determined before and during the immunization period at weeks 7 and 17 and at week 107, which is 89 weeks following infection. The assay was performed as previously described [10-12] with HCVpp expressing E1 and E2 envelope glycoproteins of the isolates CG (J) (AF333324) and UKN1B 12.6 (AY734975)

Virus quantification

Quantification of HCV RNA in serum was monitored using Amplicor (Roche Diagnostics, Basel, Switzerland) [13].

Cellular immune responses.

The number of HCV specific PBMCs was determined by lymphoproliferation and ELISPOT assay at regular time points during the immunization period and after experimental HCV exposure as previously described [8].

Analysis of HCV E1 And E2 sequence diversity

Viral RNA was isolated at weeks 1, 3, 6, 12, 31, 36 and 41 weeks after challenge for as long as the virus was detectable in serum, making use of the QIAamp Viral RNA Mini Spin protocol (Qiagen Benelux B.V., Venlo, The Netherlands). DNA synthesis and outer PCR was performed in one step using the Qiagen One Step RT-PCR kit (Qiagen Benelux B.v., Venlo, The Netherlands), followed by a conventional nested PCR. The following primer sets were used to sequence E1; outer primer set HCPr638 (5'CTG TCY TGY TTG ACC RTC CCA GC3' (nt 544 to 566 when aligned to H77) and HCPr639 (5'CCC GTC AAC GCC GGC RAA GAG TAR C3' nt 1149 to 1125) inner

primer set HCPr640 (5'GTY TGA CCA YCC CAG CTT CCG CT3' nt 551 to 573) and HCPr641 (5'TCA ACG CCG GCR AAR AGT ARC RYC AC3' nt 1145 to 1120). To completely cover the E2 region, the following 3 primer sets were used, fragment I; outer primer HCPr642 (5'TGG CGC TAC TCT TTG CCG GCG TTG A3' nt 1121 to 1145) and HCPr643 (5'AGG CAC CTA GGT GTC AAC CA3' nt 1844 to 1825); inner PCR HCPr644 (5'CGG CGT TGA CGG GGC GAC CTA3' nt 1137 to 1157) and HCPr645 (5'CCC GAG CCA CAT TTT GTG TA3' nt 1820 to 1801) Fragment II; outer HCPr691 (5'GTG TGY GGY CCA GTG TAY TG3' nt 1510 to 1529) and HCPr692 (5'CCA CTC YGT YGT RGA CAG YA3' nt 2037 to 2018); inner HCPr693 (5'GAY GTG RTG CTY CTY AAC AA3' nt 1609 to1628) and HCPr694 (5'TCC TCC AAG TYR CAR CGC TC3' nt 1988 to 1969). Fragment III; outer HCPr646 (5'TGA TCT GCC CCA CGG ACT GC3' nt 1757 to 1776) and HCPr647 (5'TCC CGG TCC AAG GCG TAA GC3' nt 2459 to 2440); inner primers HCPr648 (5'ACG GAC TGC TTC CGG AAG CA3' nt 1768 to 1787) and HCPr649 (5'GGC GTA AGC TCG TGG TGG TA3' nt 2448 to 2429)

RESULTS

Vaccine Specific Antibody Responses

The induction of vaccine specific antibodies in serum was determined by ELISA. As shown in **Figure 1**, all four vaccinees developed envelope specific antibodies with similar magnitude and kinetics, increasing following each immunization and peaking 14 to 17 weeks following the first immunization. After experimental HCV exposure at week 18, HCV specific antibody responses gradually declined, yet persisted at low levels until the end of the study. Similar declines have been noted previously by us and others as a frequent sequelae of HCV infection in chimpanzees. Antibody formation directly after experimental infection is low and often falling below the detection limits of standard ELISA assays [14, 15]. In the control animal HCV specific antibodies were only observed after challenge (data not shown).

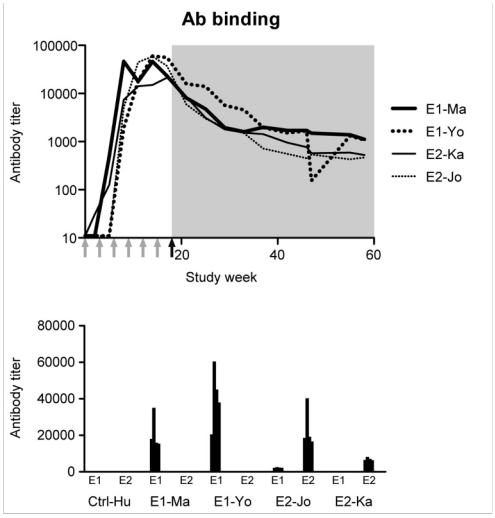


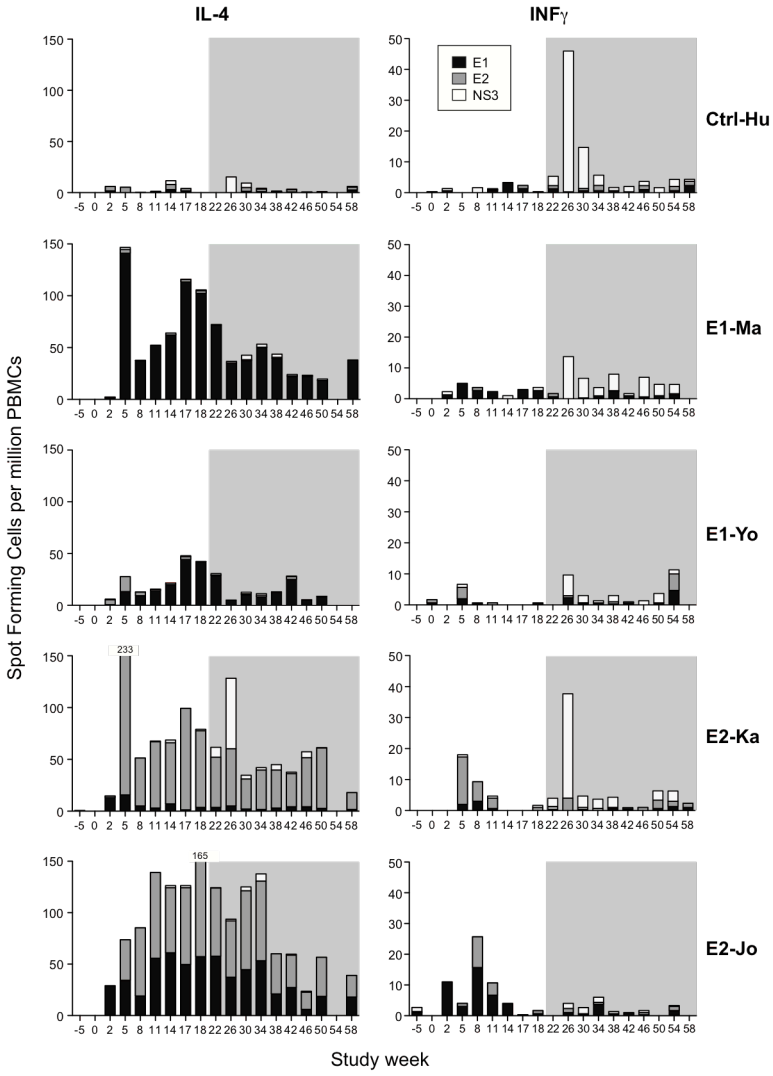
Figure 1; Humoral responses.
A) Vaccine specific antibody titers over time, i.e. E1-specific antibodies were measured in E1-Ma and E1-Yo, and E2-specific antibodies in serum from E2-Ka and E2-Jo. Time points of immunizations are indicated by grey arrows under the X-axis and the time point of challenge by a black arrow. The shaded area represents the post challenge follow up period. B) Evaluation of cross reactivity. Serum samples collected at weeks 0, 13, 14, 15 and 16 (separate bars) were tested for binding to E1 and E2 with standard ELISA

Induction of HCV specific T-cell responses by immunization

Induction of cellular immune responses against the vaccine proteins (E1 or E2) as well as an HCV protein not included in the vaccine (NS3) were assessed by lymphocyte proliferation and ELISpot analysis. As shown in **Figure 2**, both E1 immunized animals and the two E2 immunized animals developed vaccine- induced IL4 secreting cells (140 IL4 sfu/10^6 PBMCs in E1-Ma, 44 IL4 sfu/10^6 in E1-Yo, 217 IL4 sfu/10^6 in E2-Ka and 106 IL4 sfu/10^6 in E2-Jo). Instead, the number of vaccine induced IFNγ secreting cells was much lower in all four animals (<10 sfu/10^6 PBMCs in each animal). These data implied a Th2-dominant immune response, which correlated with high vaccine induced antibody levels in serum at the time point of challenge. As

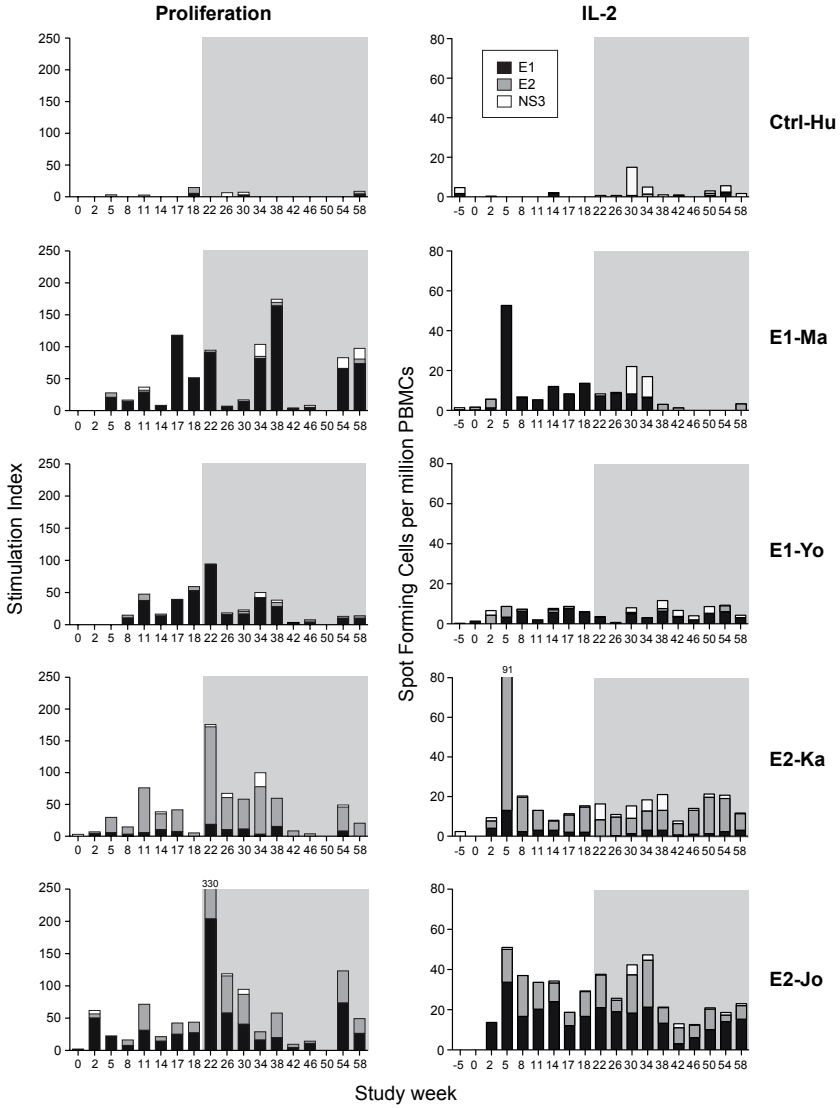
expected only weak cellular immune responses were observed against the non-vaccine viral protein NS3 following HCV exposure in all 5 animals.

Figure 2; HCV-specific IL4 and IFNγ responses



Magnitude over time of IL4 and IFNγ cytokine responses of individual animals were measured against recombinant proteins HCV E1 (black bars), E2 (grey bars) and NS3 (white bars). Responses are shown for the two E1 protein immunized animals E1-Ma and E1-Yo, the two E2 protein immunized animals E2-Ka and E2-Jo and the naive control animal Ctrl-Hu. Immunizations were given at weeks 0, 3, 6, 9, 12 and 15 followed by HCV exposure at week 18. The shaded area represents the post challenge follow up period. Shown are the numbers of antigen specific spot forming cells per million PBMCs minus the background (mean of triplicate medium controls).

Figure 3; HCV-specific lymphoproliferation and IL2 responses.



Magnitude over time of lymphproliferation and IL2 cytokine responses of individual animals were measured against recombinant proteins HCV E1 (black bars), E2 (grey bars) and NS3 (white bars). Responses are shown for the two E1 protein immunized animals E1-Ma and E1-Yo, the two E2 protein immunized animals E2-Ka and E2-Jo and the naive control animal Ctrl-Hu. Immunizations were given at weeks 0, 3, 6, 9,

Expectedly, NS3 specific responses were IFNγ biased while the envelope specific responses preserved their Th2-like characteristics. T-cell responses were further substantiated by lymphoproliferation and L-2 ELIspot **(Figure 3)**. Although, E2-Jo was only immunized with recombinant E2 protein and not with E1, *in vitro* stimulation of PBMCs with E1 also induced cytokine production. This cross-reaction between the two envelope proteins was also observed in the humoral response **(Figure 1B)**. Serum samples collected at weeks 0, 13, 14, 15 and 16 from E2-Jo were found to have some reactivity against E1, which was low compared to the E2 binding. Neither E1 nor E2 specific T-cell responses were observed prior to challenge in Ctrl-Hu.

Neutralizing capacity of vaccine-induced antibodies

To determine if the antibodies induced by the vaccine proteins, E1 or E2, were functional in neutralizing HCV, pseudoparticle assays (HCVpp) were performed on serum obtained before and 7, 17 and 107 weeks following initial immunization. The HCVpp were pseudotyped with E1E2 glycoproteins derived from strain UKN1B 12.6, which has a closely related amino acid sequence to the vaccine strain, or CG (J), an isolate most similar to the challenge inoculum [16] As a control, the glycoprotein from RD114, the cat endogenous virus, was used. **Figure 4** displays the percentage of target cells infected by HCVpp, and the inhibition of infection by serum from vaccinees obtained at different time points. Comparison of the percentage of infection between the immunized animals and the control animal Ctrl-Hu, showed more efficient inhibition by sera obtained from the E1 immunized animals than by sera from the E2 immunized animals. At week 7 both of the E1-immunized animals developed antibodies that strongly inhibited CG (J) HCVpp infection ($p<0.01$ Two Way ANOVA), while no inhibition was observed by sera from the two E2 immunized animals. This CG (J) neutralization was also observed, but at a somewhat reduced level, at week 17 during the pause between immunization and viral exposure. In both E1 immunized animals, significant neutralization ($p<0.01$) was also observed against

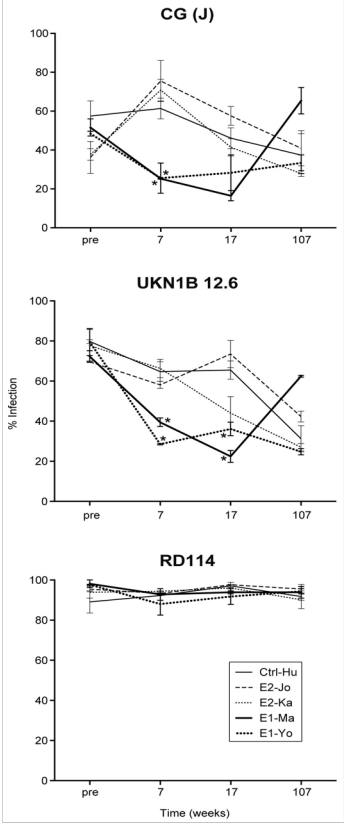


Figure 4; Neutralizing antibodies.

Serum samples collected before immunization and at weeks 7, 17 and 107 were tested for the inhibition of infection of Huh-7 target cells by HCVpp. Serum was tested against CG (J) and UKN1B 12.6 HCV isolate envelopes, while RD114, a cat endogenous virus derived envelope, served as a control. Results are expressed as percentage of infected cells Plotted in the graphs are the results of two independent assays, both performed in duplicate. * reduction of percentage of infected cells as compared to the control animal ($p<0.01$).

HCVpp exposing the UKN1B 12.6 envelope both at week 7 and 17. As expected, persistent HCV infection induced neutralizing antibodies inhibiting HCVpp infection with both CG (J) as well as UKN1B 12.6 isolates (week 107), **Figure 4**. Vaccine induced levels of neutralization declined in the absence of HCV in E1-Ma and E1-Yo

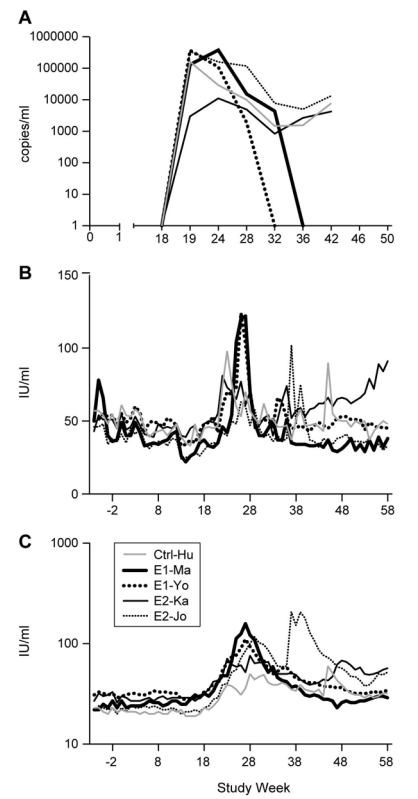


Figure 5; Viral load and liver enzymes.
Shown is; A) the plasma viral load (copies ml^{-}): B) ALT levels (IU ml^{-}) and C) γGT (U ml^{-}) in individual animals observed over time

Neutralizing antibody titers correlate with clearance of infection.

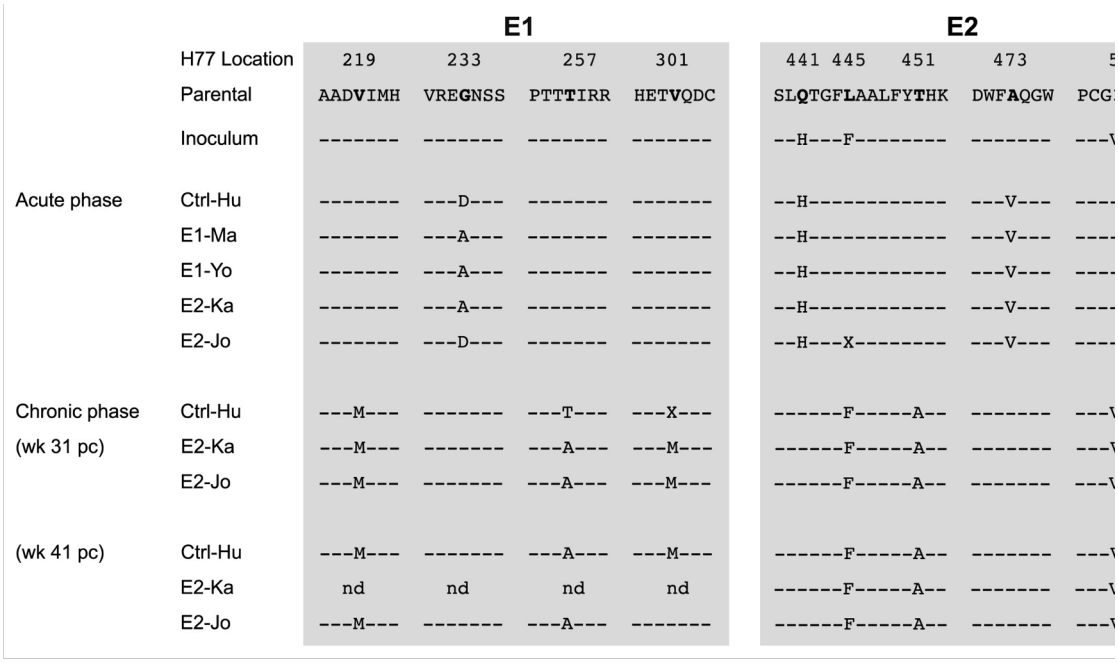
Three weeks after the final protein immunization, efficacy against viral challenge was evaluated by intravenous exposure to 100 CID HCV 1b J4.91'01. All four vaccinees and the naive control animal became acutely infected. A peak virus load between 1 and 5 x10^5 IU ml^{-1} was reached 1 to 6 weeks after challenge in all animals except E2-Ka, where the peak viremia was 1 log lower **(Figure 5A)**. Subsequently, the virus load decreased to levels between 10^3 and 10^4 IU ml^{-1} in the control and the two E2 immunized animals. Instead, in the two E1 immunized animals virus became undetectable in serum and liver biopsies by quantitative PCR 18 weeks after challenge. Furthermore, liver enzyme levels, ALT and γGT returned to pre-challenge values in these two animals **(Figure 5B and Figure 5C)**. In contrast to the E1 immunized animals, all others remained persistently infected and retained slightly elevated liver γGT **(Figure 5C)** (p=0.039, two tailed Student's t-test).

No evidence for selective immune pressure directed against envelope regions

To rule out that development of chronic infection in the E2 vaccinated animals was either due to an ineffective E2 induced vaccine response, or due to viral evolution and vaccine escape we performed longitudinal sequence analysis of the E1 and E2 regions of the predominant viral variants in plasma. Furthermore we compared these sequences to the challenge inoculum

J4.91'01 as well as a parental virus isolate from which it was derived HC-J4/91 (D10750) [17]. As shown in **Figure 6**, all the non-synonymous mutations relative to the inoculum aligned. In the acute phase only one amino acid residue change was observed in the E1 region (G232A or G232D), and three in the E2 region (F438L, A466V and V496I). These changes seem to be unrelated to the vaccination strategy, as they are observed in all animals irrespective of the antigen they were immunized with.

In the chronic phase of infection in the animals not immunized with E1, three de novo mutations occurred in putative E1 epitopes (V219M, T256A and V301M) and two in E2 (H434Q and T444A). Surprisingly, all residues which became established during the acute phase reverted to the residue found in the original inoculum. Interestingly, with the exception of position 301 in the E1 region (valine in E2-Jo and methionine in the other animals), the dominant viral variants (in both E1 an E2 regions) that emerged at the end of the study were found to be identical in the three animals that became persistently infected.



		E1				E2			
	H77 Location	219	233	257	301	441 445	451	473	5
	Parental	AADVIMH	VREGNSS	PTTTIRR	HETVQDC	SLQTGFLAALFYTHK		DWFAQGW	PCG
	Inoculum	-------	-------	-------	-------	--H---F--------		-------	---V
Acute phase	Ctrl-Hu	-------	---D---	-------	-------	--H----------		---V---	----
	E1-Ma	-------	---A---	-------	-------	--H----------		---V---	----
	E1-Yo	-------	---A---	-------	-------	--H----------		---V---	----
	E2-Ka	-------	---A---	-------	-------	--H----------		---V---	----
	E2-Jo	-------	---D---	-------	-------	--H---X--------		---V---	----
Chronic phase	Ctrl-Hu	---M---	-------	---T---	---X---	------F-----A--		-------	---V
(wk 31 pc)	E2-Ka	---M---	-------	---A---	---M---	------F-----A--		-------	---V
	E2-Jo	---M---	-------	---A---	---M---	------F-----A--		-------	---V
(wk 41 pc)	Ctrl-Hu	---M---	-------	---A---	---M---	------F-----A--		-------	---V
	E2-Ka	nd	nd	nd	nd	------F-----A--		-------	---V
	E2-Jo	---M---	-------	---A---	-------	------F-----A--		-------	---V

Figure 6; sequence analysis

Sequence alignments from the peripheral blood of the envelope regions E1 and E2 from the indicated animals following persistent viremia (viral escape or evasion). Nucleotides are represented by standard single letter codes, dashes indicate identity with the reference sequence HC-J4/91, substitutions or mutations are represented by single letter codes. Sequences were derived from time points after virus escaped the immune responses. Samples from the following time points were used to isolate viral sequences, Ctrl-Hu 41 weeks, E1-Ma 6 weeks, E1-Yo 12 weeks, E2-Ka 31 weeks and E2-Jo 41 weeks (last time-points from which amplification of sequences was routine) after HCV exposure

DISCUSSION

The partial characterization of the immune correlates of clearance of HCV infection and the identification of the targeted viral antigens, facilitated the rational development of a vaccine for the prevention of chronic HCV infection [8, 18, 19]. HCV vaccine studies directed at the induction of virus neutralizing antibodies have focused either on the use of the E1E2 heterodimer, or the E2 protein alone [2, 15, 20, 21]. However, two lines of evidence have suggested a role for E1. Firstly, broadly neutralizing human monoclonal antibodies to the E1 glycoprotein have been identified [6, 7]. Secondly, functional analysis has identified several membrane active regions in E1 (as well as E2) involved in virus-cell membrane fusion [5].

Here we tested the hypothesis that the independent use of HCV E1 subunit as a vaccine immunogen would be capable of inducing neutralizing antibodies and facilitating protection from HCV infection. Interestingly, although immunization with E1 was successful in inducing neutralizing antibodies, these did not have a directly of blunting the acute viral peak after a relatively high dose, intravenous HCV exposure. Ultimately, E1-neutralizing antibodies, together with recruited T-helper responses appear to have facilitated HCV 1b clearance. However, additional mechanisms such as antibody dependant cellular cytotoxicity (ADCC), recruited innate responses in the liver or late induction of CTL activity may have contributed to clearance.

Although antibodies were also present in the serum of the E2 immunized animals, these antibodies lacked the ability to neutralize HCV and the virus persisted in these animals. It could be speculated that the E2 protein devoid of its HVR-1 was misfolded and incapable of inducing NAbs. However, comparison of E2 proteins with and without HVR-1 for reactivity with a range of patient sera showed no clear differences, suggesting that the conformational structure of the E2 protein lacking the HVR-1 as used in this study was antigenically intact (data not shown).

While the immune responses observed in the E1 immunized animals were restricted to the vaccine-component, chimpanzee E2-Jo, did not only respond to E2 but also to E1 in both cellular as well as humoral immune assays. Although this observation is somewhat unexpected, it must be noted that shared immunoreactivity between the two envelope glycoproteins was

reported previously [22] and may be elicited in animals that carry the appropriate MHC makeup.

The majority of the HCV vaccine efficacy studies in chimpanzees were performed before the development of suitable HCV neutralization assays therefore only limited data have been available on the correlation of HCV neutralization and vaccine efficacy. Furthermore, in the majority of HCV protein vaccine efficacy studies to date, E1 and E2 were administered as heterodimer [21] and as the HCVpp also express E1 and E2, it is impossible to determine the role of the separate envelope components. This is the first study to demonstrate a direct correlation with vaccine induced E1-neutralizing antibodies and early clearance of HCV infection. Earlier studies have focused on E2 as the lead vaccine target given the observations of E2-binding inhibition assays and the characterization of several E2 specific virus neutralizing mAb [23, 24]. The E2 antigen used in this study was devoid of the HVR-1 and therefore the outcome does not allow direct comparison with earlier protein vaccine efficacy studies. Our efforts to focus the immune response to the more conserved regions of E2 failed as the antibodies generated with this strategy did not inhibit infection with HCVpp. The lack of the HVR-1 may possibly have reduced binding to one of the scavenger receptor class B type 1 (SR-B1) regions [11, 25]. Since SR-B1 also mediates HCV uptake and cross-presentation by dendritic cells in humans [26], this may have resulted in suboptimal antigen presentation and the formation of lower affinity antibodies with reduced neutralizing capacity, despite induction of high T-cell and binding antibody responses.

Following immunization, all vaccinees developed an antigen specific Th-2 dominant immune response directed towards the vaccine components as shown by increased lymphoproliferation, the high number of HCV specific IL4 secreting cells and by the formation of HCV specific antibodies before challenge. The degree of protection observed against this heterologous 1b variant is different than the complete protection obtained with E1E2 heterodimers against HCV-1a challenge of chimpanzees [15]. However, our data concur with the outcome of re-exposure to HCV in humans and chimpanzees, where viral clearance is observed much later, after the acute phase [2].Athough vaccine mediated formation of viral escape variants is of great concern , this was not observed in this study.

This proof of principle study reveals the previously unrecognized importance of E1 alone as a candidate HCV vaccine antigen. Moreover these findings suggest that further insights into the structure-function relationship of the E1E2 heterodimer are required. Improved understanding of the dynamics and structural changes upon receptor binding and membrane fusion may reveal conserved domains which could be exploited as neutralizing antibody targets for immunization.

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CONFLICT OF INTEREST

The authors declare no conflict of interest exist.

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3

Consequences Of HCV Infection; Innate Immune Responses In Chimpanzees.

3.1

Evaluation Of IL-28B Polymorphisms And Serum IP-10 In Hepatitis C Infected Chimpanzees.

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ABSTRACT

In humans, clearance of hepatitis C virus (HCV) infection is associated with genetic variation near the IL-28B gene and the induction of interferon-stimulated genes, like IP-10. Also in chimpanzees spontaneous clearance of HCV is observed. To study whether similar correlations exist in these animals, a direct comparison of IP-10 and IL-28B polymorphism between chimpanzees and patients was performed.

All chimpanzees studied were monomorphic for the human IL-28B SNPs which are associated with spontaneous and treatment induced HCV clearance in humans. As a result, these particular SNPs cannot be used for clinical association studies in chimpanzees. Although these human SNPs were absent in chimpanzees, gene variation in this region was present however, no correlation was observed between different SNP-genotypes and HCV outcome. Strikingly, IP-10 levels in chimpanzees correlated with HCV-RNA load and γGT, while such correlations were not observed in humans. The correlation between IP-10, γGT and virus load in chimpanzees was not found in patients and may be due to the lack of lifestyle-related confounding factors in chimpanzees.

Direct comparison of IP-10 and IL-28B polymorphism between chimpanzees and patients in relation to HCV infection, illustrates that the IFN-pathways are important during HCV infection in both species.

INTRODUCTION

Worldwide, an estimated 170 million people are chronically infected with the hepatitis C virus (HCV) [1] and are therefore at risk to develop liver diseases, like cirrhosis and hepatocellular carcinoma. Upon infection with HCV, only a minority of individuals can clear the virus spontaneously, while the majority of patients become chronically infected [2, 3]. In recent years, an important role for host factors has been documented in determining the progression towards chronicity of HCV as well as prediction of therapy-induced clearance of HCV [4-10]. Genetic variation on chromosome 19, within an intergenic region upstream of the IL-28B gene, encoding for the IFN lambda 3 protein (IFNλ3) correlates with both spontaneous as well as treatment-induced clearance [6, 8]. Furthermore, low plasma levels of interferon-gamma-inducible protein 10 (IP-10) prior to therapy predict treatment-induced clearance of HCV infection [7, 11-13] but whether these two factors interrelate is currently under intense debate. However, this is highly relevant since both parameters directly interfere with antiviral IFN-pathways.

Chimpanzees are not only the closest living *evolutionary* relatives of humans but also the only validated animal model to study HCV infection. Similar to humans, HCV in chimpanzees can either lead to a self-limiting infection or to viral persistence, and also in the chimpanzee, antigen specific cellular immune responses are believed to be important [14-16]. In patients, low pre-treatment IP-10 levels in serum and strong upregulation of IFN-stimulated genes (ISGs) during treatment in the liver were shown to correlate with successful IFNα based therapy [7, 9, 11, 12, 17]. However, in contrast to humans, standard IFNα-based therapy failed to reduce serum HCV RNA levels in chronically infected chimpanzees [18], and therefore mechanisms leading to treatment-induced sustained virological response (SVR) cannot be studied in these animals. The lack of clinical response to IFNα in chimpanzees was suggested to be similar to that in patients nonresponsive to therapy: a maximum induction of ISGs by HCV itself, rather than by treatment with exogenous IFNα [18].

Interestingly, also a weak association between the preferred IL-28B genotypes and low serum IP-10 baseline levels in patients chronically infected with HCV was recently reported [11, 19]. This suggests an interaction between IP-10 and IFNλ during the course of HCV infection in humans [12]. However, at present, a clear understanding of the mechanisms and their potential role in

viral clearance is lacking. In this study, we aimed to evaluate and compare IP-10 and polymorphism near the IL-28B gene in chimpanzees and humans in relation to HCV infection to obtain better insight in the potential association between the two host factors in HCV infection.

We show that, however chimpanzees do not possess the same human SNPs near the IL-28B gene, allelic variation is present in this region in chimpanzees. No association was observed between IL-28B polymorphism and the course of HCV infection in chimpanzees. Furthermore, we report a positive correlation between peripheral levels of IP-10 and HCV RNA as well as γGT levels in chimpanzees, but not in patients. These findings demonstrate differences between HCV-infected chimpanzees and patients, which may impact pathophysiologic processes in the liver.

MATERIALS & METHODS

Study population

To study the potential roles of IP-10 and polymorphisms during HCV infection in chimpanzees, we examined these parameters using the unique repository of chimpanzee DNA and serum samples of the Biomedical Primate Research Centre (BPRC). This database contains surplus material from blood samples collected routine health check purposes by both the BPRC as well as third party owners of chimpanzees. The work described here, was designed around the availability of the samples thus, no samples were collected from chimpanzees for the purpose of this work. Table 1 summarizes the relevant characteristics of the chimpanzee cohort included in this study. The animals were divided into different groups based on their HCV status: 1) “high viral load”: animals who were experimentally infected with HCV and virus load consistently higher than 200,000 IU/ml, where 200,000 is the median virus load of the animals in the study; 2) “low viral load”: animals who were experimentally infected with HCV and viral load consistently lower than 200,000 IU/ml; 3) “cleared”: animals who were experimentally infected with HCV and resolved infection 4) “naive“: non–infected animals that serve as healthy controls. In addition, DNA samples of 44 animals were screened for the presence of genetic variation near the IL28B gene. In total 63 chimpanzees were included in the analysis of the IL-28B region of which 11 animals were not part of the BPRC breeding.

Blood samples were obtained from patients with chronic HCV infection visiting the outpatient clinic of the Erasmus Medical Center (see Table 2). Patients were infected with either HCV genotype 1 (n=37), genotype 2 (n=3), genotype 3 (n=13), genotype 4 (n=4) or genotype 6 (n=1). Patients co-infected with human immunodeficiency virus, hepatitis A virus, hepatitis B virus or hepatitis D virus were excluded from the study. The protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center and all patients gave their written informed consent. In line with the chimpanzee cohort, the patients were divided into different groups based on their HCV status: 1) “high virus load”: virus load consistently higher than 500,000 IU/ml, where 500,000 is the median virus load of all patients in this study; 2) “low virus load”: patients with virus load consistently lower than 500,000 IU/ml.

Determination of HCV load and liver enzymes

Quantification of HCV RNA levels was assessed using the Cobas Amplicor HCV monitor test (Roche Diagnostics, Branchburg) according to the instructions of the manufacturer. The levels of γGT, ALT and AST were determined in serum using the COBAS Integra 400 plus analysis system.

IL-28B gene associated polymorphisms in chimpanzees

To determine the genotype of the rs8099917 and rs12979860, genomic DNA was isolated from either chimpanzee PBMC or whole blood [20] and the nucleotide sequence was determined by PCR amplification followed by direct sequencing. To assess the genotype of rs8099917, a previously described set of PCR primers was used [21], while a new set of primers was designed for the amplification of a fragment of 209 bp containing rs12979860 (IL-28-860-F2 5'-GGACAAGCGGCGCTTATCG-3' and IL-28-860-R2 5'-GGCTCCAGGTCGGG GCG-3'). To amplify the fragments of interest, 1 μg of genomic DNA, 0.5 μM of each PCR primer, 0.2 mM dNTP each, 1.5 mM $MgCl_2$ and 1 unit of Platinum Taq DNA polymerase (Invitrogen, Paisley, Scotland) were mixed in the appropriate PCR buffer in a total volume of 50 μl. The PCR conditions were: 1 cycle of denaturation at 94 ºC for 2 min, 30 cycles of denaturation at 94 ºC for 30 s, annealing at 61 ºC for 30 s and extension of 68 ºC for 30 s, followed by a final extension for 7 min at 68 ºC. PCR fragments were purified, using the GenJET™ Gel Extraction Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. The nucleotide sequence of the PCR fragments was determined using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). 0.2 μM of PCR primer, 1 μl of BigDye Terminator-mix and 5x sequencing dilution buffer in a total volume of 10 μl and sequenced directly on an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, USA). The data was analyzed using MacVector™ version 12.0.2 (MacVector, Inc Cambridge, UK). Sequences observed for rs12979860 were deposited in the EMBL database and received the accession numbers HE599784 and HE599785.

The genotype of the IL28B associated SNPs in patient blood were determined using competitive allele-specific PCR (KASP; KBioscience *Hoddesdon*, UK).

Serum levels of IP-10, IL-29 and IL-28A/B

The concentrations of IP-10, IL-29 and IL-28A/B in serum were determined using commercially available kits. The levels of IP-10 in patient- and chimpanzee sera were measured using the Quantikine Kit (R&D

Systems, Abingdon, UK; sensitivity of assay: 2 pg/ml). The concentration of IL-29 and IL-28A/B in chimpanzee sera was measured using VeriKine-DIY Human Interferon Lambda immunoassays specific for IL-29 or for the combination of IL-28A/B (both from PBL InterferonSource, NY, USA). The assays were performed according to the manufacturer's instructions, and the detection limits for both immunoassays were 62.5 pg/ml. IL-29 ELISA results were confirmed by the human IL-29 ELISA Ready-SET-Go!® kit (e-Bioscience, San Diego, USA, detection limit for this assay was 8 pg/ml).

Statistics

Statistical analysis was performed using Graphpad Prism 5.0 (GraphPad Software, Inc., La Jolla, USA). To test if a significant correlation exists between two quantitative variables, the correlation coefficient r^2 is calculated as a measure of variation of 2 variables. In addition, to test whether this correlation was significant, a t-test was performed. A two tailed T-test was used to determine statistical difference of liver-enzymes between patients and chimpanzees.

RESULTS

No evidence found for the human SNPs associated with HCV clearance in chimpanzees

In humans, spontaneous as well as treatment-induced clearance was found to be associated with a series of SNPs upstream of the IL-28B gene [4-6, 8, 10]. To assess whether clearance of HCV in chimpanzees is associated with the same SNPs, genotype analysis of chimpanzee DNA was performed for rs8099917 and rs12979860.

The SNP rs8099917 consistently showed thymidine at this position on both chromosomes, indicating that all animals tested were homozygous rs8099917-TT carriers. In humans rs8099917-T is the preferred allele associated with HCV clearance. In addition, the chimpanzees were also genotyped for the rs12979860 SNP, and again no evidence was found for variance at this position. All animals tested had homozygous rs12979860-TT genotypes. In humans, the rs12979860-T allele is the so-called risk-allele associated with an increased risk for viral persistence. Thus, both IL-28B associated human SNPs are not present in chimpanzees.

Chimpanzees possess a unique sequence near the IL-28B gene

Since rs8099917 and rs12979860 genotyping was performed by sequencing, the neighboring nucleotide sequences are also known, allowing us to compare this region with documented human genome sequences (National Institutes of Health, Bethesda, USA). Two different haplotypes were observed near rs12979860, HE599784 and HE599785. Notable was the strong uneven distribution: 62 out of 63 animals were heterozygous, carrying both HE599784 and HE599785. Only one animal was found to be homozygous for HE599785. These data suggest that an as yet unknown balancing selection may be operative on this region.

No detectable IFNλ in serum from chimpanzees

In humans carrying the rs12979860-TT genotype, significant lower serum levels of IFNλ were documented in chronically infected patients as compared to patients with resolved infections [19, 22]. To investigate whether this difference could also be observed in chimpanzees, ELISA was performed on sera from chronically infected animals and animals that cleared the infection. Using commercially available immunoassays designed to detect human IL-28A/B and IL-29, we were unable to detect IFNλ in sera from any of the

chimpanzees tested, neither in animals chronically infected with HCV nor animals that spontaneously cleared the infection. In the same assays, IL-28A/B and IL-29 could be detected in sera from patients with chronic HCV infection (data not shown; detection limit of the assays: 62.5 pg/ml and 8 pg/ml, respectively).

IP-10 levels in serum correlate with viral load in HCV infected chimpanzees, but not in humans

To investigate whether the IP-10 levels in serum were associated with viral clearance, we compared IP-10 levels of chronically infected animals with a high HCV load (>200,000 IU/ml), low HCV load (<200,000 IU/ml), chimpanzees that had resolved HCV infection early after experimental exposure, and a group of HCV-naïve, non-exposed chimpanzees. As shown in **Figure 1A**, the IP-10 levels in sera of animals with a high viral load were significantly higher relative to animals in the other groups. The serum IP-10 levels of animals with low viremia, animals that resolved the infection and

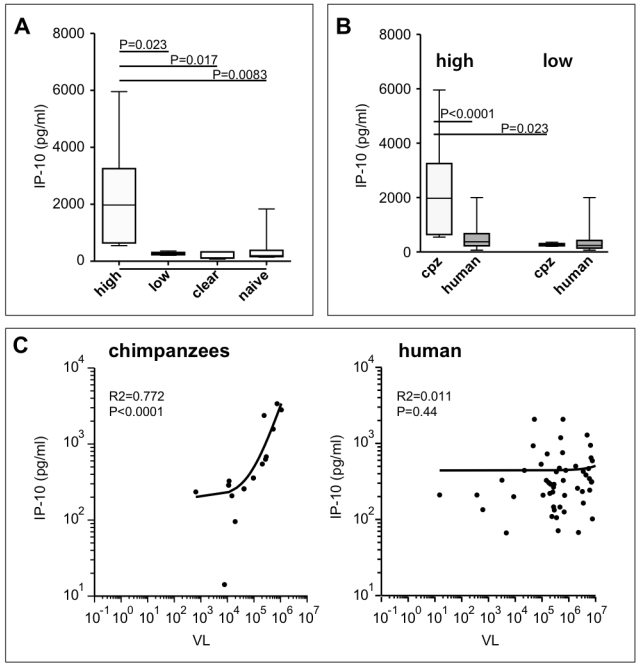


Figure 1; **Correlation between HCV viremia and IP-10 in serum in chimpanzees**
Box-whisker plots indicate the interquartile range and the median (horizontal line) of IP-10 concentrations in serum from animals of the different groups; "high HCV load" where the virus load of individual animal is higher as compared to median value of 200,000 IU/ml; "low HCV load" where the virus load of individual animal is lower as compared to the median value of 200,000 IU/ml; HCV resolvers and naïve, non-exposed (A). Box-whisker plots indicate the interquartile range and the median (horizontal line) of IP-10 concentrations in serum of humans and chimpanzees with high HCV load (> median virus load) and low HCV load (< median virus load) (B) and IP-10 concentrations plotted against HCV-RNA load in serum from chimpanzees and humans (C). A significant correlation between the two parameters is defined as $r^2>0.85$ and $p<0.05$ where "r^2" is a measure for correlation and "p" is a measure for the quality if this correlation.

HCV-naïve animals were relatively low and comparable between these groups.

Earlier studies in chronically infected patients demonstrated an association between viral load and IP-10 levels in serum [23, 24]. Therefore, we evaluated whether this correlation also existed in chimpanzees. As shown in **Figure 1B**, at a low viral load, the IP-10 levels were similar in humans and chimpanzees. In contrast, serum IP-10 levels were higher in chimpanzees with a high viral load relative to human patients with similar high viral loads. A strong correlation between serum IP-10 concentration and viral load was observed in chronically infected chimpanzees. In contrast to chimpanzees, neither a correlation was observed in the overall group of patients chronically infected with HCV (**Figure 1C)**, nor when this group was divided into subgroups according to their rs12979860 genotype (data not shown).

Serum IP-10 concentrations correlate with γGT in HCV infected chimpanzees, but not in humans

In patients, the concentration of the serum aminotransferase ALT is used as indicator of active damaging processes in the liver during chronic HCV infection. However, in chimpanzees, γGT tends to be more increased rather than ALT. We therefore assessed whether IP-10 levels in blood correlated with the liver enzymes γGT, ALT and AST in chronically HCV infected chimpanzees and patients. As shown in **Figure 2A**, in chimpanzees the serum levels of IP-10 strongly correlated with γGT (r^2=0.91 and p<0.0001), while no correlation was observed between IP-10 and

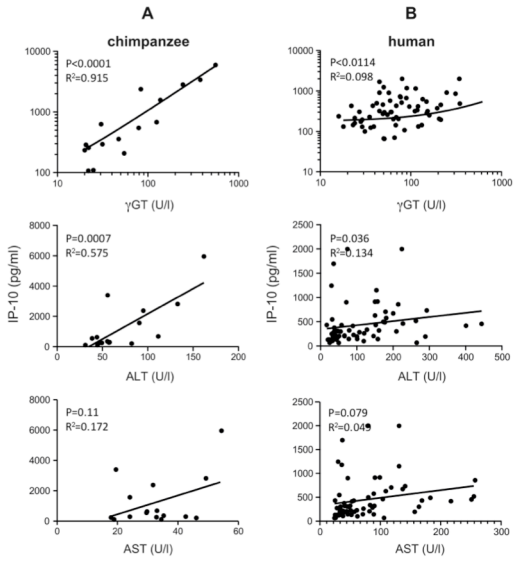
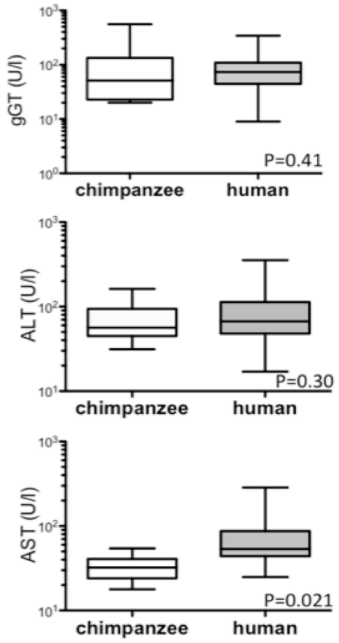


Figure 2; **Relation between IP-10 levels and aminotransferases in serum**
Relation between IP-10 and liver enzymes γGT, ALT and AST from HCV-infected individual chimpanzees (A) and HCV-infected patients (B) A significant correlation between the two parameters is defined as r^2>0.85 and p<0.05 where "r^2" is a measure for correlation and "p" is a measure for the quality if this correlation.

either ALT (r^2=0.58; p=0.0007) or AST (r^2=0.17; p=0.11). In contrast, in HCV infected patients, nocorrelations were observed between serum IP-10 and the aminotransferases **(Figure 2B)**. Direct comparison of the levels of ALT and AST in serum of patients and chimpanzees demonstrated that only moderate ALT values were observed in chimpanzees, whereas 4 out of 58 patients exceeded an ALT value of 200 U/ml which is more then 4 times the maximum reference value for healthy humans and chimpanzees **(Figure 3)**. Similarly, also AST levels were only moderately increased in chimpanzees, whereas 11 patients exceeded the maximum reference value by threefold. In contrast, serum γGT levels were found to be substantially elevated in some individuals from both species: in 3 out of 16 chimpanzees and 7 out of 60 patients, the γGT levels exceeded 200 U/ml, which is more then 5 times its reference value in healthy humans and chimpanzees.

Figure 3; γGT, ALT and AST in humans and chimpanzees chronically infected with HCV
Interquartile range and the median (horizontal line) of gGT, ALT and AST in U/ml in chimpanzees and patients chronically infected with HCV.



DISCUSSION

In humans, HCV clearance is associated with specific IL-28B gene polymorphisms as well as the levels of specific ISG, such as IP-10 [4, 5, 7, 8, 10, 13]. It has been suggested that these pathways are associated with one another in humans [11, 19] although the interrelating mechanism is still unknown. The work presented here was designed to assess whether a potential correlation between IFNλ and polymorphism near the IL-28B gene can be studied in chimpanzees.

In humans, genetic variation near the IL-28B gene is associated with spontaneous as well as treatment-induced clearance of HCV [4, 5, 8, 10]. In chimpanzees, we found no evidence for these human SNPs and therefore it is unlikely that these specific SNPs play a role during HCV infection in chimpanzees. Instead, all animals tested were found to be homozygous carriers for rs12979860-TT and rs8099917-TT, which is in line with recently reported data of chimpanzees from another primate center [25]. A potential explanation may be that HCV is regarded as a human disease as no documentation is available on wild chimpanzees infected with HCV. In that respect different evolutionary selective pressure may have caused differences in innate responses between both species.

Since the documented SNPs, rs12979860 and rs8099917 are located in an intergenic region rather than within gene-encoding regions of the DNA, there may be a link between the region where the SNPs are located and another, yet unspecified gene. Given the fact that humans and chimpanzees share a common ancestor, the chimpanzee sequence likely represents the ancestral genotypes. This is in line with the finding that Central and Western African human populations carry the rs12979860-TT genotype at high frequency [10].

Even though humans and chimpanzees show 98.8% identity at the DNA level [26], many SNPs identified in humans are not necessarily present in chimpanzees. Although no evidence was found in chimpanzees for the two SNPs with documented relevance during HCV infection in humans, additional polymorphism near the IL-28B gene was detected. In the animals tested, a remarkable level of heterozygous carriers was observed, this may suggest a balancing selection being operative on this region. Our findings cannot be explained by the breeding strategy as concluded on the basis of variation in

the mtDNA [27], and because it was also observed in animals from outside the BPRC breeding colony. The underlying mechanism responsible for this heterogeneity to assure a genetically diverse population is unknown.

In serum from chimpanzees no IFNλ was detected, and therefore no conclusion could be drawn from its effect on the outcome of HCV infection in chimpanzees. IFNλ has been measured in chimpanzees before using human detection reagents [25]. Given the rapid normalization of IFNλ levels in chimpanzees shortly after infection, it is expected that IFNλ is too low to detect during the chronic phase of infection. Lower IFNλ levels in chimpanzees could not be explained by a different copy number of the IFNλ-encoding genes as both in humans as well as chimpanzees only one copy of each of the IL-29/IL28A and IL-28B genes was observed (human and chimpanzee database supported by National Institutes of Health, Bethesda, USA).

In patients chronically infected with HCV, low baseline IP-10 levels are predictive for successful treatment-induced clearance [7]. Our data show that IP-10 levels are higher in chronically infected chimpanzees relative to patients. This finding is in line with the suggestion that HCV infection causes stronger upregulation of ISGs in chimpanzees as compared to humans [18]. Furthermore, based on data from a limited number of animals with high HCV-RNA levels, it was suggested that the failure of chimpanzees to respond successfully to IFN-based antiviral treatment was due to high baseline activation of the IFN-system. Based on our data it is to be expected that peripheral IP-10 levels from the animals in the Lanford-study are high. Given the documented correlation between high IP-10 and limited treatment success in humans, it is tempting to speculate that chimpanzees with a high HCV load and subsequently high baseline IP-10 levels in serum are indeed equivalent to human non-responders to IFN-based therapy. However, this implies that animals with lower IP-10 may respond to IFN-based therapy.

As humans and chimpanzees do not to show the same variation near the IL-28B gene, we were not able to confirm the earlier documented association between IL-28B variation and γGT levels in humans [17]. We did however find a correlation between γGT, IP-10 and virus load. This may imply, interrelating mechanisms play a role in both humans and chimpanzees, but that species-specific factors may contribute to biochemical differences between both species.

Our data show that although chimpanzees do not possess the SNP near the IL-28B gene that are associated with HCV outcome in humans, chimpanzees do show genetic variation in this region. Furthermore, we found a positive correlation between IP-10 and viral load as well as γGT in chimpanzees, which was not found in patients. This difference may reflect the heterogeneous characteristics of HCV induced reactions in the liver of both species. The correlation between IP-10, virus load and γGT may reflect the lack of confounding factors in chimpanzees, since heavy alcohol intake, diabetes and obesity are known to influence the progression of hepatitis C virus infection in humans, but not in chimpanzees.

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TABLES

Table 1
Characteristics of chimpanzee population

	genotype	yrs pi	HCV load	IP-10	ALT	AST	γGT
High virus load							
n=8	1	4 (4-21)	505000 (207000-1080000)	2247 (547-5956)	91 (39-162)	34 (20-54)	204 (30-558)
Low virus load							
n=6	1	3 (2-4)	31908 (649-95500)	274 (209-359)	58 (46-82)	33 (18-46)	33 (20-55)
Cleared HCV infection							
n=6	1	4.5 (3-10)	0	143 (71-131)			
Naïve HCV							
n=10			0	414 (146-1834)			

Relevant characteristics of the chimpanzee population studied, the HCV-genotype, the time since HCV exposure, HCV-RNA load (IU/ml), IP-10 (pg/ml) levels in peripheral blood, the liver enzymes ALT (U/ml), AST (U/ml) and γGT (U/ml). Shown are the average values and the minimal and maximal values.

Table 2
Overview of the characteristics of the patient population, chronically infected with HCV

patient ID	genotype	HCV load	IP-10	ALT	AST	γGT
High virus load						
n=30	1,2, 3 and 6	7827576 (585185-42100000)	540 (67-2000)	135 (17-444)	80 (25-251)	87 (16-347)
Low virus load						
n=27	1, 3 and 4	255115 (15-2114180)	281 (66-902)	84 (20-263)	64 (24-255)	70 (9-205)

Relevant characteristics of chronic HCV patients studied: HCV genotype, HCV-RNA load (IU/ml), IP-10 (pg/ml) levels in peripheral blood, the liver enzymes ALT (U/ml), AST (U/ml) and γGT (U/ml). Shown are the average values and the minimal and maximal values.

3.2

Increased Soluble CD14 Levels In The Absence Of Liver Fibrosis And Microbial Translocation In Hepatitis C Virus Infected Chimpanzees.

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ABSTRACT

Increased levels of soluble C14 (sCD14) in serum may either be the consequence of increased microbial translocation or diminished clearance of bacterial products by hepatic dysfunction. During HIV/SIV infection sCD14 is up-regulated as a consequence of pathological disruption of the gut epithelial barrier resulting in increased microbial translocation. Also in HCV infected patients with advanced liver fibrosis, increased levels of sCD14 have been reported. Since the liver plays an important role in clearance of translocated bacteria, fibrosis may negatively affect clearance and thus contribute to higher sCD14 levels. We have tested the hypothesis that increased levels of sCD14 in blood, during chronic HCV infection, can occur in the absence of hepatic fibrosis and microbial translocation.

In chimpanzees and human patients, sCD14 was quantified as a marker for systemic microbial exposure, and intestinal fatty acid binding protein (I-FABP) as a marker for gut perturbation. In addition, LPS-binding-protein, EndoCab, sIL-2R and IL-6 were analysed.

HCV infected patients with advanced liver fibrosis exhibit increased levels of sCD14. In the majority of these patients up-regulation of CD14 could not be explained by microbial translocation, as shown by normal levels of endocyte necrosis. This finding was confirmed in HCV infected chimpanzees. Also in these animals, up-regulated sCD14 levels were observed in the absence of I-FABP. In addition, as chimpanzees do not develop hepatic fibrosis, sCD14 elevation cannot be explained by impaired hepatic clearance of microbial products. These data support the hypothesis that replicating HCV can cause up-regulation of sCD14 production.

INTRODUCTION

Soluble CD14 (sCD14) levels in blood and microbial translocation are often used to describe the same pathological process. However, this is not entirely correct. Microbial translocation is the process of leakage of microbial products from the gastrointestinal tract to extra-intestinal locations like mesenteric lymph nodes, peripheral blood or the liver. Increased exposure to microbial products then leads to increased levels of sCD14 in the blood. However, increased levels of sCD14 may also be a consequence of diminished clearance of bacterial products by hepatic dysfunction. Hence, a more precise term for sCD14 up-regulation would be 'microbial exposure' as schematically explained in **Figure 1**.

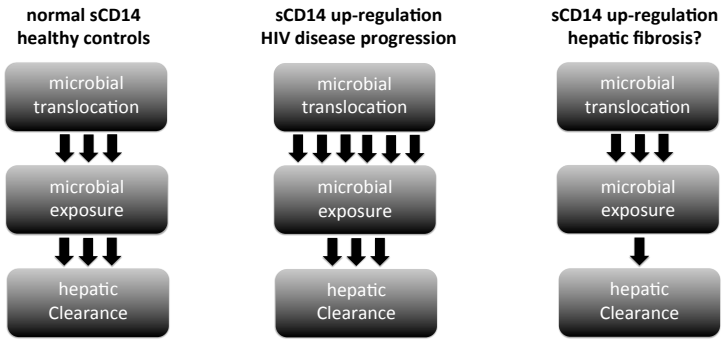

Figure 1; Schematic model of the term microbial exposure.
Systemic microbial exposure is determined by the balance between microbial translocation and hepatic clearance. During HIV infection perturbation of the gut causes more microbial products to enter the blood. During HCV infection translocation is innitialy not affected and increased microbial exposure is caused by decreased hepatic clearance hepatic clearance.

In HIV infected humans, increased levels of sCD14 in serum have been described in relation to disease progression [1, 2]. sCD14 up-regulation was associated with increased levels of intestinal-fatty-acid-protein (I-FABP), which is released during endocytic cell death [1, 2]. As a consequence of enterocyte death, the integrity of the gut epithelial barrier is lost, which gives rise to increased microbial translocation. Sustained increased microbial translocation and subsequent microbial exposure, may result in chronic systemic immune activation, ultimately leading to immune exhaustion and loss of immune function [3].

In HCV infection, elevated sCD14 levels are associated with hepatic fibrosis [4, 5] and HCV seroconversion [6] but not with increased levels of LPS-

binding protein (LBP) [6]. Although it is assumed that portal hypertension negatively affects the capacity of the liver to clear residues from translocated bacteria, the exact mechanism is still unknown [7]. Here, we tested the hypothesis that HCV-induced sCD14 up-regulation can occur in the absence increased microbial translocation and hepatic fibrosis.

In order to address the effect of HCV infection and liver fibrosis on sCD14 levels, we have included chimpanzees into the study. Chimpanzees are the only validated animal model to study HCV infection. Like humans, infection of chimpanzees can either lead to a spontaneously resolved infection or the animals become persistently infected. Unlike in humans, hepatic fibrosis as a result of HCV infection has not been documented in chimpanzees [8]. Previously, we reported on the correlation between the soluble activation marker interferon gamma inducible protein 10 (IP-10) and HCV load in chimpanzees [9]. These data indicate that immune activation does take place in the absence of immune-pathology in HCV-infected chimpanzees. Therefore, HCV infected chimpanzees offer the unique opportunity to investigate whether systemic microbial exposure and immune activation can occur in the absence of liver fibrosis.

Several assays have been described to quantify microbial translocation, systemic microbial exposure and immune activation in peripheral blood. Lipopolysaccharide (LPS), a glycolipid of the outer cell wall of gram-negative bacteria, elicits an inflammatory reaction via TLR4 triggering that is required when bacterial products are present in peripheral blood. However, direct quantification of LPS in stored peripheral blood is technically complicated and data are unreliable when samples have been in contact with polypropylene [10]. Since all samples used in this study had been stored in polypropylene ampoules, quantification of LPS was not the preferred technique. Therefore, a panel of surrogate markers was used for quantification of systemic microbial exposure. LPS-binding protein (LBP) is predominantly produced by hepatocytes and in order to initiate monocyte activation, LBP chaperones LPS to membrane CD14 (mCD14) and TLR4. To prevent monocyte overstimulation, sCD14 is being produced in the liver. Similar to mCD14, sCD14 can bind the LPS-LBP-complex. However, sCD14 is a non-signaling molecule, a so-called decoy receptor. Both LBP and sCD14 can be measured in blood and are indirect but widely-used markers for LPS-induced monocyte activation and systemic microbial exposure [6, 11-

[13]. In addition, quantification of EndoCab, antibodies that are directed against the core antigen of LPS [6, 13], was used.

Enterocytes are part of the intestinal mucosa. When the mucosa is damaged, intestinal fatty acid protein (I-FABP) is released by enterocytes in peripheral blood. I-FAPB is used as an indicator for damage to the gut mucosa [4, 13] and is a measure for microbial translocation. Immune activation was quantified by measurement of soluble IL-2R (sIL-2R or sCD25) and IL-6 levels as described earlier [4, 13].

To aim of this study was to investigate whether microbial exposure during chronic HCV infection is a consequence of increased microbial translocation or from reduced microbial clearance due to fibrosis. In humans with advanced fibrosis, increased microbial exposure was observed as shown by elevated levels of sCD14. This was however not associated with increased levels of I-FABP, indicating that sCD14 was not a result of increased microbial translocation. To assess if hepatic fibrosis was the cause of increased microbial exposure, sCD14 levels were determined in chimpanzees as a model for HCV infection without fibrosis. Also in chimpanzees increased microbial exposure was observed, indicating that liver fibrosis is not the sole cause of sCD14 up-regulation. By contrast, in HIV/SIV-infected chimpanzees, up-regulation of sCD14 was caused by microbial translocation as shown by the higher I-FABP levels.

MATERIALS & METHODS

Bio-repository

Samples were collected from chronic HCV patients visiting the outpatient clinic of the Erasmus MC, Rotterdam, and stored at -80°C. Relevant clinical and virological details of the individual patients are presented in Table 1. The institutional ethical review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals. Stage of liver fibrosis was determined by METAVIR score on core needle biopsies, and HCV RNA load was determined with COBAS Taqman assay. Patient samples were divided into two groups patients with little or no fibrosis (F0/F1), and patients with advanced fibrosis (F3/F4).

Samples from chimpanzees used in this study were collected for reasons of health and welfare management or obtained from the BPRC repository. Plasma and serum samples were stored in polypropylene tubes (Greiner Bio-One) analyzed in parallel no difference was observed between the two sample types (data not shown).

Liver tissue samples were collected post mortem. As chimpanzees have never been euthanized in the setting of experimental work conducted at the Biomedical Primate Research Centre (BPRC), liver material was collected from animals that had died from natural causes. Based on the HCV status, chimpanzees were divided into different groups: 1) ‘‘naïve”: non–infected animals (n=7) serving as healthy controls: 2) Acute (n=10), <6 weeks after experimental HCV infection; 3) ‘‘low virus load’’: HCV infected animals (n=5) with a virus load consistently lower than 250,000 IU/ml; 4) ‘‘high viral load’’: HCV infected animals (n=5) with a virus load consistently higher than 250,000 IU/ml; 5) ‘HIV/SIV’; (n=19) chimpanzees that were infected with HIV and/or SIV for more than 4 years.

All HCV chimpanzees were experimentally infected with HCV genotype 1 and HCV RNA load was determined with a ROCHE HCV Taqman assay. Relevant parameters of patients and chimpanzees are summarized in Table 1.

Assessment of liver fibrosis

Liver tissue was fixed in 10% formalin, processed by conventional methods and embedded in paraffin. Four micrometer sections were prepared and stained with hematoxylin and eosin using standard procedures. Subsequently, sections were microscopically examined by an experienced

pathologist for signs of hepatic fibrosis like disruption of liver parenchyma architecture, extension of fibers, formation of large fibrous septa, pseudolobe separation, collagen deposition and the presence of inflammatory cells.

Quantification of systemic bacterial exposure and immune activation.

Commercially available ELISA kits were used to quantify the levels of sCD14 (R&D Systems, Minneapolis, MN, USA), I-FABP, LBP and EndoCab (all from Hycult Biotech, Plymouth Meeting, PA, USA), IL-6 (Ucytech, Utrecht, The Netherlands) and sIL-2R (eBioscience, Inc, San Diego, CA, USA) in stored plasma or serum. All tests were performed according to the manufacturer's guidelines. The assays were performed in duplicate and data were analyzed using ADAMSEL software (BPRC, Rijswijk, The Netherlands).

Statistical analysis

To determine if there were significant differences between the different study groups, either unpaired parametric or non-parametric tests were performed (Prism 6.0.). Statistical significant differences were defined as $p<0.05$.

RESULTS

No signs of liver fibrosis in chimpanzees

In the hepatic tissue from adult chimpanzees, >10 years after HCV genotype 1 infection, only minor lymphocyte infiltrates were found, mainly located around the portal ducts but also dispersed throughout the liver. No signs of vascular construction were observed and the hepatic bile ducts were regularly shaped. Furthermore, no steatosis was observed. The data suggest that no fibrosis was present in the livers of chimpanzees.

Increased sCD14 is associated with HIV and HCV infection

In patients with advanced fibrosis (F3/F4), the levels of sCD14 were significantly higher (p=0.021) compared to healthy controls, median 4774 ng/ml (ranging from 2624 to 6785 ng/ml) and 1712 ng/ml (ranging from 1084 to 7328 ng/ml), respectively **(Figure 2A)**. Chimpanzees overall had lower sCD14 levels compared to humans **(Figure 2B)**. Yet, a modest but significant difference was observed between naïve and HCV infected chimpanzees with a high virus load (p=0.032) and with HIV/SIV infected chimpanzees (p=0.0014). No association was observed with acute HCV infection in chimpanzees or with low virus load.

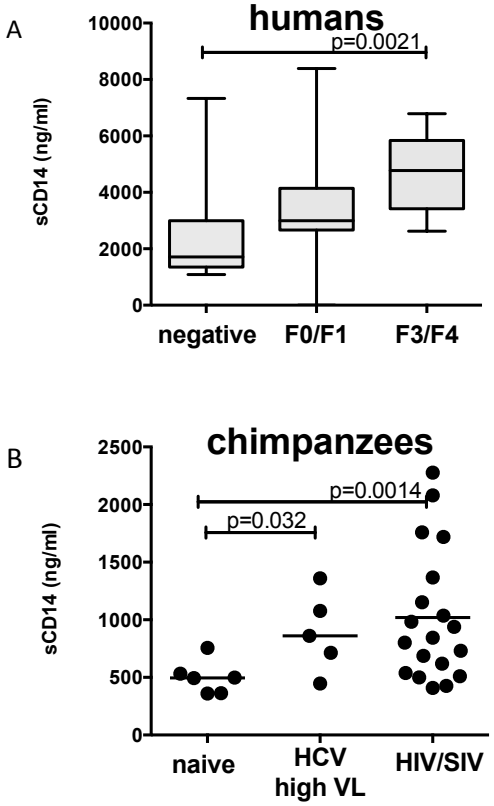

Figure 2; Soluble CD14 expression in peripheral blood in humans and chimpanzees.
sCD14 levels in plasma/serum was analyzed in **(A)** negative healthy human controls, patients with fibrosis stage F0 or F1 and patients with advanced fibrosis, stage F3 or F4. (B) Naïve chimpanzees, HCV infected animals with consistently high virus load and HIV/SIV infected chimpanzees The boxes indicate the median with 25th and 75th percentile, and the whiskers the minimum and maximum values. P-values were calculated using unpaired two-tailed t-tests,

Signs of disrupted gut barrier integrity after HIV not HCV infection

To investigate if elevated sCD14 was caused by disruption of gut epithelial cells, I-FABP was quantified. No association was observed between I-FABP and fibrosis in HCV infected patients **(Figure 3)**. Also in HCV infected chimpanzees the gut epithelial barrier remained intact as shown by the low levels of I-FABP on serum.

By contrast, increased levels of I-FABP were detected in HIV/SIV infected chimpanzees compared to chimpanzees with high HCV load ($p<0.0001$), chimpanzees with low HCV load ($p<0.0001$), and animals with acute HCV infection ($p=0.009$). Due to two outliers, no significant difference was reached between the naïve animals and HIV/SIV infected animals.

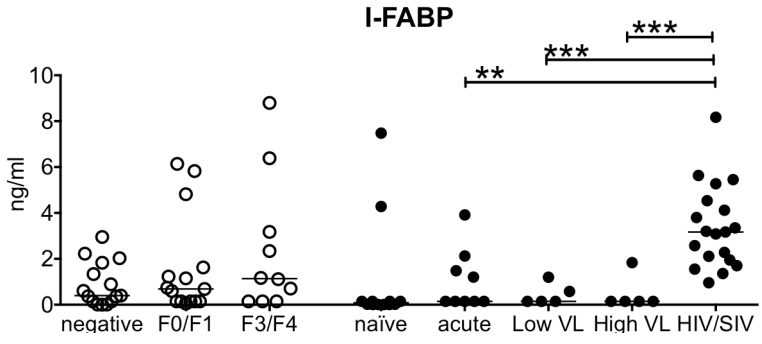

Figure 3; I-FABP levels in peripheral blood I-FABP in human negative, F0/F1 and F3/F4) (open dots) and chimpanzees (naïve, acute HCV infection, low HCV load, high HCV load and HIV/SIV) (black dots). Lines represent the mean values. P-values were calculated using the non-parametric, unpaired, two tailed t-tests

Increased LBP in HIV infected chimpanzees

In addition to I-FABP, also EndoCab and LBP have been reported to correlate with microbial translocation and HIV disease progression [2]. To investigate if these markers are associated with HCV infection, their levels in serum were quantified. No correlation was found between hepatic fibrosis and EndoCab and the development of fibrosis **(Figure 4A)** or LBP **(Figure 4B)**. Similarly, no difference was observed between levels of EndoCab or LBP in HCV infected chimpanzees **(Figure 4A and 4B)**. By contrast, a modest increase was observed in LBP in serum from HIV/SIV infected chimpanzees compared to naïve animals ($p=0.034$) **(Figure 4B)**. This observation is in line with the up-regulated sCD14 in HIV/SIV infected chimpanzees.

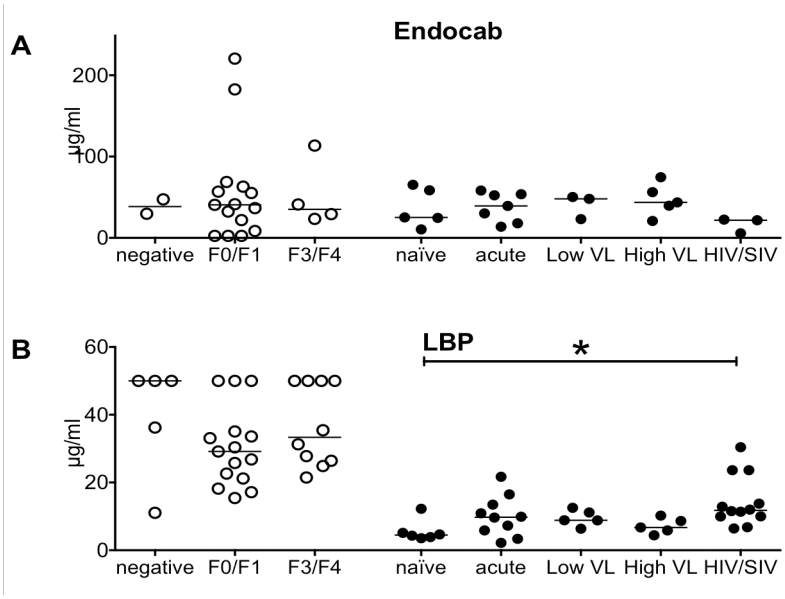

Figure 4; Levels op biomarkers in peripheral blood

Levels of EndoCab (A), LBP (B), in humans (open dots) and chimpanzees (black dots). Analyzed were HCV negative human controls, chronic HCV carriers with F0/F1, and patients with F3/F4. Naïve chimpanzees were compared to animals with acute HCV infection, chronic HCV infection with low virus load, chronic HCV infection with high virus load and HIV.SIV infection. Lines represent the mean values. P-values were calculated using a non-parametric, unpaired, two tailed t-tests.

No evidence for systemic immune activation in HCV-infected chimpanzees

To assess if HCV infection led to increased systemic immune activation, IL-6 and sIL-2R levels were determined. No differences in IL-6 and sIL-2R levels were found between healthy controls and patients No evidence was found for up-regulated IL-6 or sIL-2R HCV or HIV/SIV infected chimpanzees (data not shown).

DISCUSSION

In concordance with literature [8], no indication was found for hepatic fibrosis in HCV infected chimpanzees by histological examination of liver sections.

Our data show an association between increased sCD14 levels and advanced hepatic fibrosis in humans. These data are in accordance with data published by Sandler et al. [4]. Elevated levels of sCD14 are indicative for increased systemic bacterial exposure, which can be caused by increased microbial translocation from the gut, or by reduced clearance of bacterial products by the liver. As described earlier, in a fraction of the HCV patients with advanced hepatic fibrosis, evidence for loss of gut epithelial barrier integrity was found [4]. Also in our study cohort, some individual patients showed elevated I-FABP levels. These data suggest that severe fibrosis may corroborate sCD14 up-regulation by microbial translocation. This is in line with the observation that I-FABP levels were not increased in HCV infected chimpanzees, who typically do not develop fibrosis.

As shown by the elevated sCD14 levels in chimpanzees with high HCV load, sCD14 can also be up-regulated during HCV infection in the absence of fibroses and increased microbial translocation. *Ex vivo* studies have shown that human hepatocytes produce sCD14 [14]. However, studies in the chimeric mouse model demonstrated that HCV infection does not promote sCD14 production by human hepatocytes [15]. Hence, this does not exclude that other cells in the liver, like monocytes or epithelial cells, may be responsible for sCD14 up-regulation in response to HCV infection [14].

Excessive microbial translocation is seen as a characteristic AIDS in humans and is not observed in non-pathogenic SIV infections in sooty mangabeys or African green monkeys [16]. Although HIV does generally not cause AIDS in chimpanzees, some cases of pathogenic infections with HIV in chimpanzees have been reported [17-19]. In the HIV/SIV-infected animals tested here, modest but significantly higher levels of sCD14 were observed in HIV/SIV infected chimpanzees compared to naïve animals. In addition, higher levels of I-FABP were found in these animals. This indicates that increased levels of sCD14 after HIV/SIV-infection are caused microbial translocation as a result of endocyte apoptosis. Without AIDS-like disease in these chimpanzees, the loss of epithelial integrity in the gut is similar to what

is seen in HIV patients progressing to AIDS, and macaques infected with pathogenic SIV-strains [2, 12, 20]. Th17 cells in the gut mucosa are the primary target cells for HIV and SIV in humans and macaques [21, 22]. Depletion of these cells are thought to lead to imbalanced regulation and impaired epithelial barrier function [23]. It would therefore be interesting to study whether Th17 cells are targeted by HIV or SIV in chimpanzees. In this light, the lack of association between HCV infection and microbial translocation is not surprising. In contrast to HIV, primary target cells of HCV are located in the liver and not in the area where microbial translocation takes place. There is evidence for the presence of HCV proteins in enterocytes, albeit this is not accompanied by inflammatory responses [24] and therefore, pathophysiologic changes in the mucosal barrier are not likely to occur.

The sCD14 levels in chimpanzees are much lower compared to humans. We acknowledge that the sCD14 levels detected in healthy control animals are different from the levels that were recently reported by Greenwood et al [25]. This difference may be explained by the individual animals that were included. In our experiments, the relatively low levels of sCD14 were confirmed by low levels of LBP. Even upon HIV/SIV infection, up-regulation of sCD14 and LBP was only marginally in chimpanzees. At present, we have no clear explanation for this. It has been described that baseline levels of microbial translocation vary between species and the environment in which one lives [26, 27]. This may indicate that the innate immune system in chimpanzees is less susceptible to activation by bacterial products compared to humans. However, we have previously shown that monocyte-derived dendritic cells in chimpanzees are susceptible to LPS stimulation [28]. On the other hand, pre-exposure to LPS may lead to tolerance to subsequent LPS stimulation [29].

IL-6 and IL-2R in peripheral blood was variably up-regulated in humans, independent from HCV infection or hepatic fibrosis. In addition, these markers were poorly expressed and not up-regulated during HIV or HCV infection. This suggests that IL-6 and IL-2R are not the appropriate markers to study HCV-induced activation. Alternatively, other activation markers, like IDO [30] and IP-10 [9]. have been described in HCV-infected chimpanzees. As expression of IP-10 correlated with HCV virus load in chimpanzees, with γGT, ALT and liver fibrosis in humans [9]. These markers may be more appropriate to study HCV induced immune activation.

In conclusion, HCV infected patients with advanced liver fibrosis exhibit increased levels of sCD14. In the majority of these patients up-regulation of CD14 cannot be explained by microbial translocation, as shown by normal levels of endocyte necrosis. This finding was confirmed in HCV infected chimpanzees. Also in these animals, up-regulated sCD14 levels were observed in the absence of I-FABP. In addition, as chimpanzees do not develop hepatic fibrosis, sCD14 elevation cannot be explained by impaired hepatic clearance of microbial products. These data support the hypothesis that replicating HCV can cause up-regulation of sCD14 production.

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Table 1

Chimpanzees; High HCV load (gt* 1)

	weeks pi†	VL‡(IU/ml)	γGT§	AST‖	ALT#
Ph-05	1144	506,000	160.6	24.5	108.0
To-05	988	250,000	480.0	50.7	177.5
Fe-05	520	384,000	65.5	26.4	91.9
Ma-05	520	1,160,000	157.1	61.1	148.7
Pe-05	1144	740,000	531.2	19.9	78.6

Chimpanzees; Low HCV load (gt* 1)

	weeks pi†	VL‡ (IU/ml)	§GT§	AST‖	ALT#
Ir-06	162	650	14.3	38.1	21.1
Fu-06	310	33,400	33.2	86.5	66.3
Jo-00	343	202,000	83.7	55.1	52.2
Hu-06	30	73,700	37.2	26.0	51.5
Ka-00	29	133,000	72.8	92.0	68.0

Chimpanzees; Acute

	weeks pi†	VL‡ (IU/ml)	γGT§	AST‖	ALT#
Iri-03	1	79,950	26.5	52.7	73.0
Ju-03	1	314,500	33.1	22.3	55.9
Ke-03	1	125,125	54.2	18.7	51.3
To-03	1	74,775	35.1	26.6	46.5
Wi-03	1	307,375	21.6	19.2	55.7
Fau-00	3	5,000	38.0	ND[h]	54.0
Li-00	3	500	29.0	ND[h]	44.0
Fua-00	5	10,000	58.0	ND[h]	50.0
Hu-00	2	100,000	24.0	16	54.0
Fua-00	2	10,000	36.0	ND[h]	25.0

Table 1 continue

Chimpanzees; Healthy controls

	weeks pi[†]	VL[‡] (IU/ml)	γGT[§]	AST[‖]	ALT[#]
Ir-03a		0	31.0	59.6	58.3
Ke-03		0	37.7	13.4	32.9
Ju-03		0	28.4	20.5	47.6
An-92		0	ND[g]	ND[g]	ND[g]
Re-92		0	ND[g]	ND[g]	ND[g]
Fr-03		0	ND[g]	ND[g]	ND[g]
Os-95		0	ND[g]	ND[g]	ND[g]

Patients; Low fibrosis

	weeks pi[†]	VL[c] (IU/ml)	γGT[§]	AST[‖]	ALT[#]
X-104		5,960	53	38	41
X-182		226	15	19	10
X-150		55,600	16	25	27
X-756		6,730	20	35	62
X-748		23,500	19	57	109
X-033		475,000	59	156	54
X-577		569,000	35	82	121
X-160		591,000	18	28	38
X-168		1,180,000	42	41	45
X-672		1,290,000	133	34	34
X-785		1,310,000	23	45	78
X-436		475,000	97	34	21
X-428		387,000	189	84	137
X-116		287,000	23	29	18
X-078		237,000	37	42	52

Table 1 continue

Patients; High fibrosis

	weeks pi[†]	VL[c ‘](IU/ml)	γGT[§]	AST[‖]	ALT[#]
X-153		362,000	67	51	77
X-551		272,000	78	85	46
X-481		362,000	119	46	60
X-722		535,000	109	51	100
X-345		544,000	103	107	98
X-317		587,000	352	250	200
X-779		972,000	253	150	253
X-774		1,090,000	22	45	47
X-227		1,560,000	48	63	80
X-553		1,930,000	231	27	43

Table 1; Individual characteristics

[*]gt, HCV genotype, [b] pi, weeks post experimental HCV infection, [c] VL, HCV load (copies/ml), [d] γGT, gamma glutamyl transpeptidase levels in blood, [e] AST, aspartate aminotransferase, [f] ALT, aspartate aminotransferase [g] ND, not determined

3.3

Spontaneous And NCR Mediated Cytotoxicity Are Effector Functions Of Distinct NK Subsets In HCV Infected Chimpanzees.

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ABSTRACT

In humans, CD16 and CD56 are used to identify functionally distinct NK subsets. Due to ubiquitous CD56 expression, this marker cannot be used to distinguish between NK cell subsets in chimpanzees. Therefore functional analysis of distinct NK subsets during HCV infection has never been performed in these animals. In the present study an alternative strategy was used to identify four distinct NK subsets on the basis of the expression of CD16 and CD94. The expression of activating and inhibiting surface receptors showed that these subsets resemble human NK subsets. CD107 expression was used to determine degranulation of the different subsets in naïve and HCV infected chimpanzees.

In HCV infected chimpanzees increased spontaneous cytotoxicity was observed in $CD94^{high/dim}CD16^{pos}$ and $CD94^{low}CD16^{pos}$ subsets. By contrast, increased NCR mediated degranulation in the $CD94^{dim}CD16^{neg}$ subset was demonstrated after NKp30 and NKp44 triggering. Our findings suggest that spontaneous and NCR mediated cytotoxicity are effector functions of distinct NK subsets in HCV infected chimpanzees.

INTRODUCTION

NK cells are important immune cells in the first line of defense to several viral infections. NK cells have ambiguous functions: they have the ability to lyse infected target cells without prior activation, and they exert regulatory functions by modulating downstream adaptive immune responses [1, 2]. NK cell activation is regulated via activating and inhibiting natural cytotoxicity receptors (NCRs). Many studies have reported on dysbalanced NCR expression on NK cells in HCV infected humans [3-13], but the results are contradictory with regard to which NCRs are associated with functional changes after HCV infection.

On the basis of the expression of CD56 and CD16, different NK cell subsets can be identified in human peripheral blood [1, 14-16]. The NK subsets have different functional properties: $CD56^{dim}CD16^{pos}$ are predominantly cytotoxic against antibody opsonized target cells [1], whereas $CD56^{bright}$ NK cells show less cytolytic activity and predominantly produce cytokines [17-19]. However, others have shown that both subsets can produce IFNγ, albeit with different kinetics [20].

The chimpanzee is the only validated animal model for the characterization of innate and adaptive immune responses during HCV infection [21-23]. Similar to humans, chimpanzees either clear HCV shortly after infection or become persistently infected. Since acute HCV infection is generally not accompanied with clinical signs, early interactions between virus and host are difficult to study in humans. An animal model circumvents these challenges, and enables experimental infection under controlled conditions with regard to HCV genotype, dose and route of infection. Although microarray analysis of an HCV infected chimpanzee has shown up-regulation of NK-associated genes [24], chimpanzee NK cell functions after HCV infection have been poorly studied. The aim of the current study was therefore to test the hypothesis that cytotoxic capacity of the NK subsets is different between naïve and HCV infected chimpanzees.

Phenotypical and functional analyses of chimpanzee NK subsets are complicated by ubiquitous CD56 expression on NK cells. Therefore, CD56 cannot be used to distinguish different NK subsets in chimpanzees [25, 26]. To be able to study NCR mediated cytolysis on different NK subsets in chimpanzees, we use here a new strategy that identifies distinct NK

subpopulations on the basis of CD94 and CD16 expression. Surface expression of CD94 identifies a functional intermediate between human $CD56^{bright}$ and $CD56^{dim}$ NK-cell subsets [17]. We report that on the basis of CD94 and CD16 coexpression 4 subsets of CD3-CD14- chimpanzee lymphocytes can be distinguished. All four subpopulations have a unique NCR expression pattern, which confirms their NK cell identity.

MATERIALS & METHODS

Phenotypic analysis of NK cells

Peripheral blood mononuclear cells (PBMCs) from naïve and HCV (n=15) infected chimpanzees were used. HCV chimpanzees (n=15) had been experimentally infected with HCV genotype 1a or 1b and HCV RNA load was determined with Roche Cobas Amplicor [27]. Virus load in serum of the animals varied between 6667 and >850,000 IU/ml. All chimpanzees had alanine-aminotransferase (ALT) values within normal limits for chimpanzees (1-140 U/l), except one animal who had an ALT of 149 U/L .PBMCs were isolated 4 weeks to 20 years after HCV infection. The original studies from which cell specimens used in the current study had been archived, were reviewed and approved by the BPRC animal experimental committee. PBMCs were isolated from heparinized blood (Vacurette, Greiner Bio-One) by standard density gradient centrifugation (Lympho Separation Medium, MP Biomedicals, OH, USA) and subsequently frozen in DMSO with 20% fetal calf serum (Life Technologies).

Phenotypic analysis of NK cells

Archived PBMC samples were thawed using standard procedures. Multiparameter flow cytometric analysis was used for phenotypical analysis of the cells. To distinguish between live and dead cells, cells were stained with the fixable Live/Dead marker (Life Technologies). Next, CD107-APC (clone H4A3, BD Biosciences) was added and after incubation, cells were stained for the surface markers CD3-PerCP (clone SP34.2, BD Biosciences), CD8-AmCyam (clone SK1, BD Biosciences), CD14-ECD (clone RMO52), CD94-FitC (clone HP-3D9), CD16-APC-H7 (clone 3G8, BD Biosciences), NKp30-PE (clone Z25, Beckman Coulter), NKp44-PE (clone Z231, Beckman Coulter), NKp46-PE (clone BAB281, Beckman Coulter), NKG2A-PE (clone Z199, Beckman Coulter), NKG2D-PE (clone ON72, Beckman Coulter), DNAM-PE (clone DX11, R&D Systems). After washing, the cells were fixed and permeabilized with perm/fix buffer (BD Biosciences) and intracellular cytokines was stained with IFNγ-AF700 (clone B27, BD Biosciences) and TNFα-PE-Cy7 (clone MAb11, BD Biosciences). After overnight fixation in 2% paraformaldelhyde solution, cells were measured on an LSRII FACS machine (BD Biosciences). Cells were analyzed with FlowJo software (version 9.8.5, Tristar; Ashland, Oregon, USA).

Reversed ADCC assay

Reversed ADCC assay was performed as previously described [3]. Fcγ-receptor carrying P815 mastocytoma cells (kindly provided by prof Laman) were used as target cells, and K562 myeloma cells were used as positive controls. In brief, $0.1*10^6$ PBMCs were resuspended in medium (RPMI 1640 medium (Life Technologies) supplemented with 10% foetal calf serum (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), 0.1 μg/ml streptomycin, (Life Technologies), 0.1 μg/ml IL-21 (PeproTech, Rocky Hill, NJ, USA) and 200 IU IL-2 (Proleukin, Chiron, Emeryville, CA, USA)) and co-cultured with target cells (E:T ratio 1). Cultures were set up with or without IgG monoclonal antibodies specific for NKp30 (clone Az20), NKp44 (clone Z231), NKp46 (clone Bab281), NKG2A (clone Z199), NKG2D (clone BAT221) and DNAM (clone F22), all kindly provided by prof. L Moretta, Instituto Giannina Gaslini, Genova, Italy. After 3 hrs incubation, cells were stained as described above.

Statistical analysis

Statistical analyses were performed using the unpaired t-test in Graph-Pad Prism software, p value ≤0.05 was considered significant..

RESULTS

Four different NK subsets in chimpanzees.

Multiparameter flow cytometric analysis was used to identify different subsets. Viable large granular cells, negative for the expression of CD3 were selected for further analysis **(Figure 1)**. Next, CD14 negative cells were analyzed for the expression of CD16 and CD94. Four distinct populations were distinguished: $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$, $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ cells.

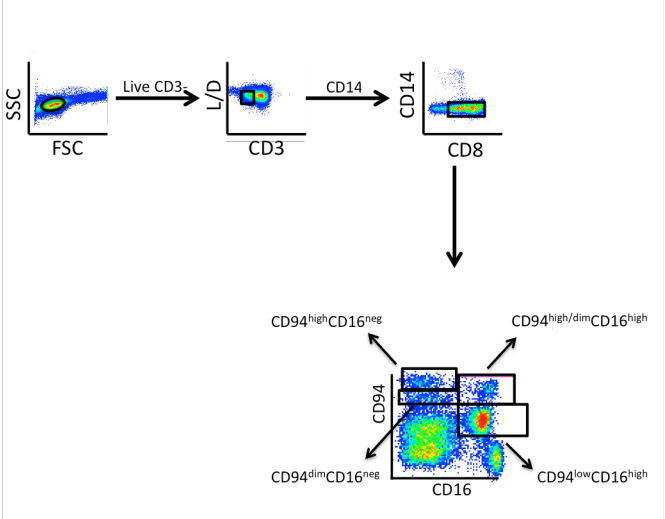

Figure 1; Multiparameter flow cytometric analysis of NK subsets.
(A) Gating strategy; large granular cells were analyzed for the expression of CD3. $CD3^{neg}$ cells were selected while $CD14^{pos}$ monocytes were excluded from further analysis. Next, CD94 expression was plotted against CD16 and gates were placed to identify $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$, $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ subsets.

Figure 2A shows the differential expression of the NCRs NKp30, NKp44, NKp46, NKG2A, NKG2D and DNAM in the four populations. Little or no NKp30 and NKp44 expression was observed on any of the four subsets directly after thawing, while expression of NGK2D was restricted to the CD16 negative populations. The inhibitory receptor NKG2A was abundantly expressed on the surface of the $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$ and $CD94^{high/dim}CD16^{high}$ cells, but not on $CD94^{low}CD16^{high}$ cells. The low expression of NKG2A may raise doubts on whether this subpopulation comprises indeed NK cells, but intermediate expression of levels NKp46 and DNAM were found. In addition, the expression of NKp46 and NKG2D shows a statistically significant up-regulation after culturing the cells in the presence of IL-2 and IL-21 prior to FACS analysis ($p<0.0001$ and $p=0.0025$, respectively)

(Figure 2B). Based on these data we conclude that the CD94^{low}CD16^{high} fraction represents an NK subset.

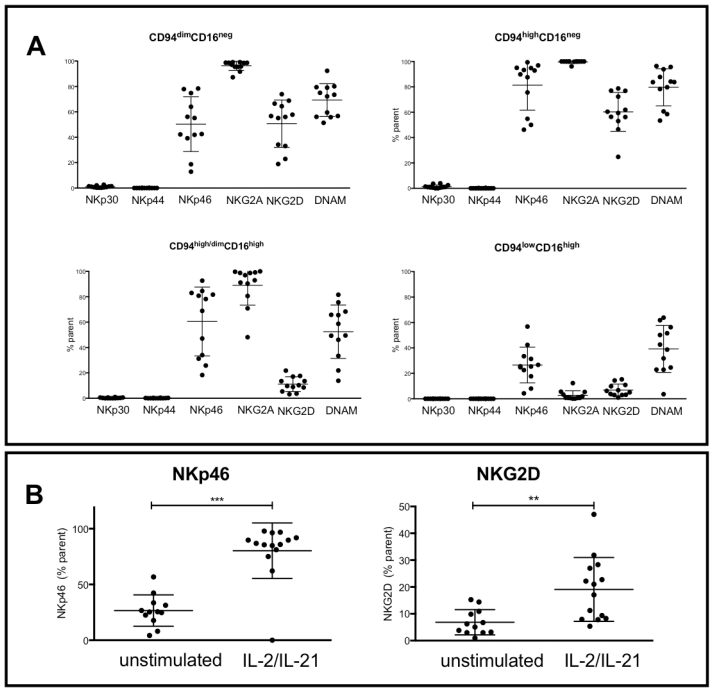

Figure 2; Frequency of NCR expression on chimpanzee-NK subsets. (A) The frequency of NKp30, NKp44, NKp46, NKG2A, NKG2D and DNAM expressing cells was calculated as the percentage of CD94^{dim}CD16^{neg} (upper left) CD94^{high}CD16^{neg} (upper right), CD94$^{high/dim}$CD16^{high} (lower left) and CD94^{low}CD16^{high} (lower right). (B) cytokine-induced up-regulation of NKp46 (left graph) and NKG2D (right graph) in CD94^{low}CD16^{high}. The dots represent the individual chimpanzees tested. Statistical significant differences are indicated by * (p value between 0.05 and 0.001, ** (p value between 0.001 and 0.0001) and *** (p≤0.0001). For every group, the bars represent the mean value and the standard deviation. Statistical significant differences are indicated and considered significant when $p<0.05$.

The phenotypical analysis strongly suggests that CD94^{dim}CD16^{neg}, CD94^{high}CD16^{neg}, CD94$^{high/dim}$CD16^{high} and CD94^{low}CD16^{high} represent NK subsets in the chimpanzee. Next, we investigated the impact of HCV infection on NCR expression by these subsets. Compared to naïve chimpanzees, HCV infected animals showed disparate deviations in NCR expression (**Supplementary Figure 1).** In the CD94^{dim}CD16^{neg} subset we observed significant up-regulation of the expression of NKp46, whereas expression of NKG2D and DNAM was down-regulated. The CD94^{high}CD16^{neg} subset showed increased levels of NKp46 and lower levels of DNAM. In the two CD16^{high} populations, the effect of HCV infection was less pronounced with small differences in NKp30, NKp44 and NKp46 expression.

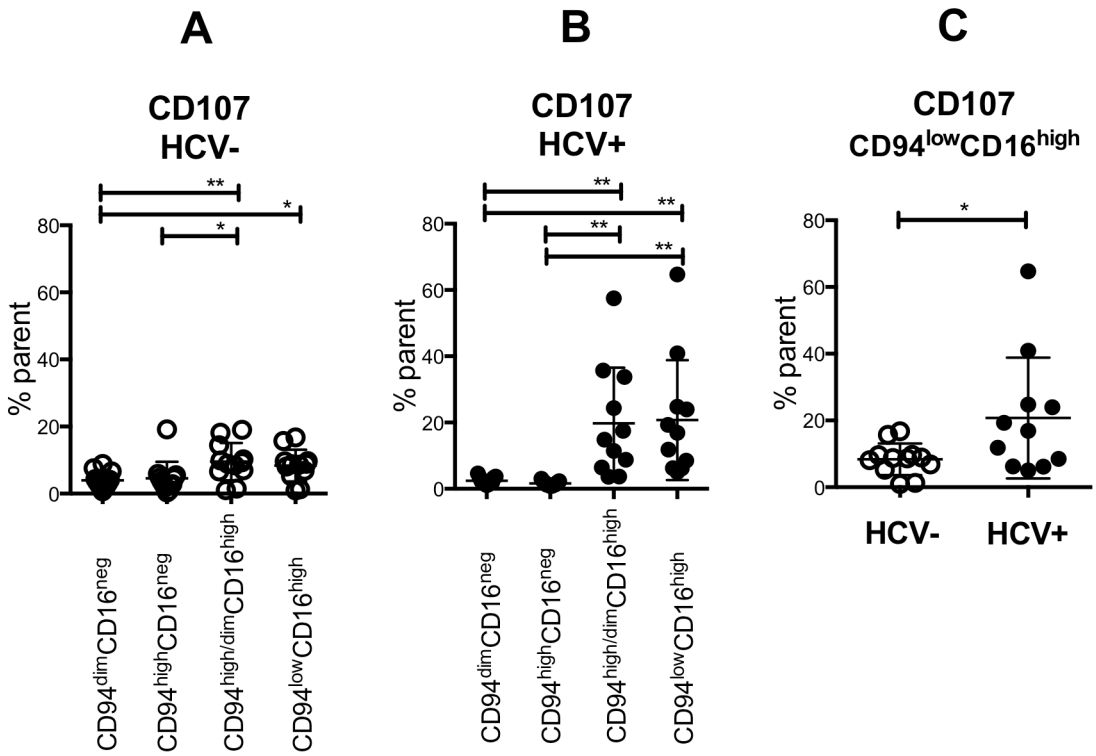

Figure 3; Frequency of CD107 expression on NK subsets from naïve and HCV infected chimpanzees.

The frequency of CD107 was calculated as the percentage CD107 expressing cells within the $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$, $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ subsets in (A) naïve and (B) HCV infected chimpanzees. (C) frequency of CD107 within the $CD94^{low}CD16^{high}$ subset in naïve (open circles) and HCV infected (black dots) chimpanzees. The dots represent the individual chimpanzees tested. Statistical significant differences are indicated as * (p value between 0.05 and 0.001, ** (p value between 0.001 and 0.0001) and *** ($p \leq 0.0001$). For every group, the bars represent the mean value and the standard deviation

Increased CD107 expression in $CD94^{low}CD16^{high}$ subset in HCV infected chimpanzees

Next, we determined intracellular CD107 levels in the different subsets as a validated marker for cytolytic capacity. In healthy controls, $CD94^{dim}CD16^{neg}$ and $CD94^{high}CD16^{neg}$ subsets expressed lower levels CD107 compared to $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ (**Figure 3A**). In HCV infected chimpanzees also higher CD107 expression was observed in the $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ subsets (**Figure 3B**). When CD107 expression was compared between naïve and HCV infected animals, significantly higher expression was observed in the $CD94^{low}CD16^{high}$ subset,

but not in CD94^{dim}CD16^{neg}, CD94^{high}CD16^{neg} or CD94$^{hig\ h/dim}$CD16^{high} cells **(Figure 3C**). Together, these data suggest that in chimpanzees CD16^{high} NK cells likely play a more prominent role Fcγ-mediated cytotoxic responses than CD16^{neg} NK cells.

HCV promotes reversed ADCC in CD94^{dim}CD16^{neg} cells

For the investigation of NCR-mediated responses, reversed ADCC assays were performed. All four subsets displayed increased CD107 expression in response to NCR-specific IgG monoclonal antibodies **(Figure 4)**, but their response to individual NCR triggering differed. Overall the response of the CD94^{low}CD16^{high} subset to NCR triggering was weaker compared to the other subsets. Especially, NKp46 triggering induced disparate degranulation in the individual subsets. While the CD94^{high}CD16^{neg} subset showed strong elevation of CD107 expression after NKp46 triggering, CD94^{low}CD16^{high} failed to respond. When comparing reversed ADCC activity between naïve chimpanzees and HCV infected animals, only few differences were observed. In HCV infected animals, the CD94^{dim}CD16^{neg} subset promoted higher degranulation after NKp30 and NKp44 triggering as compared to cells obtained from naive chimpanzees. These data suggest that HCV infection does not impair ADCC activity in chimpanzees.

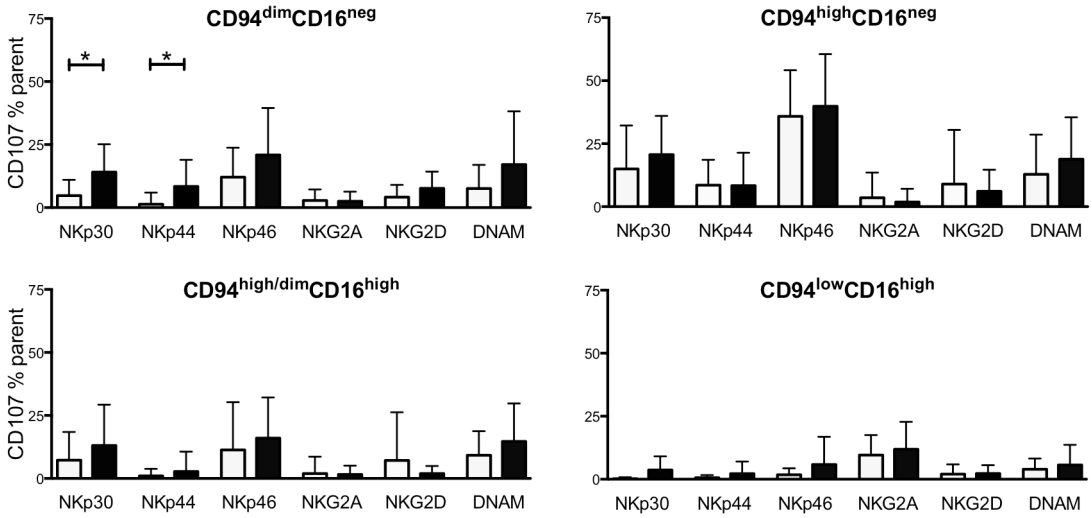

Figure 4; CD107 induction by reversed ADCC assay.

The frequency of CD107 expressing cells was calculated as the percentage of $CD94^{dim}CD16^{neg}$ (upper left), $CD94^{high}CD16^{neg}$ (upper right), $CD94^{high/dim}CD16^{high}$ (lower left) and $CD94^{low}CD16^{high}$ (lower right) in naïve (open bars) HCV infected chimpanzees (black bars) after 3 hours culture with specific IgG-antibodies binding NKp30, NKp44, NKp46, NKG2A, NKG2D and DNAM. For every group, spontaneous CD107 induction was subtracted. The bars represent the mean value and the standard deviation. Statistical significant differences are indicated as * (p value between 0.05 and 0.001), ** (p value between 0.001 and 0.0001) and *** ($p \leq 0.0001$).

DISCUSSION

The aim of this research was to develop a convenient strategy for phenotypical characterization of functionally distinct NK subsets. In humans CD16 and CD56 are used to identify functionally distinct NK subsets. As the ubiquitous high expression of CD56 precludes usage of this marker, an alternative strategy was used to separate the $CD3^{neg}CD14^{neg}$ cells into different subsets: $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$, $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ cells.

In humans and non-human primates, the vast majority of the functional NK cells express NKG2A on their surface. NKG2A expression on the $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$ and $CD94^{high/dim}CD16^{high}$ subsets confirms that these subpopulations are indeed NK cells. The lack of NKG2A expression raised doubts on whether the $CD94^{low}CD16^{high}$ subset indeed represents NK cells. However, in humans NKG2A is normally expressed on $CD56^{bright}$ and $CD56^{dim}$ NK cells. Others have reported on a $CD56^{-}CD16^{-}$ NK cell subset in non-human primates [28, 29]; this NK subset has no known counterpart in humans. However, both Pereira et al [28] and Reeves et al [29] defined NK cells as $CD3^{-}CD8\alpha\alpha^{+}CD20^{-/dim}NKG2A^{+}$, thus including NKG2A in the selection criteria. Therefore, it is unclear whether also $NKG2A^{-}$ NK cells exist. Given the fact that surface expression of CD94 identifies intermediate NK stages [17], it is tempting to speculate that the $CD94^{low}CD16^{high}$ subset represents an NK subset that is more differentiated compared to the human $CD56^{dim}$ subset. This is in line with the loss of NKG2A on the surface of fully differentiated NK cells [30].

The variable surface expression of the different NCRs on the distinct NK subsets suggests functional heterogeneity between chimpanzee NK subsets. Indeed, intracellular CD107 staining indicated that cytolytic capacity was stronger in the $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ subsets compared to the $CD94^{dim}CD16^{neg}$ and $CD94^{high}CD16^{neg}$ subsets. This is in line with studies in humans, which showed that cytolytic effector function of NK cells is strongly associated with the expression of CD16 [15, 16]. By contrast, NCR-mediated CD107 expression was poorly induced in the $CD94^{low}CD16^{high}$ subset. Reversed ADCC does not depend on CD16 expression but on the triggering of the NCR on the NK cell. However, these results should be interpreted with caution since the outcome of the experiment is highly dependent on the binding affinity of the anti-NCR antibody to the NCR. When the binding affinity of the Fc-tail of the antibody to the Fc-gamma receptor on

the target cell exceeds the affinity of the NCR-epitope on the NCR on the NK cell, the assay measures ADCC and not reversed ADCC activity. In that case the CD107 expression should be interpreted as background in the assay. Collectively, it is tempting to speculate that $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$, $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ resemble human $CD56^{dim}$, $CD56^{high/dim}$, $CD56^{neg}CD16^{high}$ and $CD16^{high}$-fully differentiated NK cells, respectively.

When comparing the cytolytic capacity of the different NK subsets between naïve and HCV infected chimpanzees, we found increased CD107 expression in $CD94^{low}CD16^{high}$ in HCV infected animals. In humans, others have demonstrated an association between increased killing capacity of $CD56^{pos}$ NK cells and protection from HCV infection [31]. In chimpanzees, this specific NK subset could not be studied but the functional resemblance may suggest that $CD56^{pos}$ cells are the human counterpart of $CD94^{high}CD16^{neg}$ in chimpanzees. In the animals studied, no increase of CD107 expression was observed in the $CD94^{high}CD16^{neg}$ cells and this may explain why the virus persisted and the animals became chronically infected.

Differences in NCR expression on the different subsets were observed between naïve and HCV infected chimpanzees. These differences were however not translated into altered NCR-mediated cytotoxicity. In the chimpanzees tested, increased NKp30 and NKp44 mediated cytolysis of $CD94^{dim}CD16^{neg}$ cells was demonstrated. This contrasts with the decreased NCR mediated target cell killing by peripheral NK cells observed by others [5]. In addition, we found no evidence for increased usage of NKG2D receptor pathways [8], as NKG2D was down-regulated on the $CD94^{dim}CD16^{neg}$ and $CD94^{high}CD16^{neg}$subsets in HCV infected animals. Decreased levels of NKG2D have been described in the livers of patients chronically infected HCV [6]. Similar observations by Pembroke et al [6], we observed that NKG2D down-regulation was accompanied by increased spontaneous degranulation. However, blood NK cells, but not liver NK cells, were studied in chimpanzees, and decreased expression of NKG2D was only observed in the $CD94^{dim}CD16^{neg}$ and $CD94^{high}CD16^{neg}$ subsets, whereas spontaneous cytolysis was an effector function of the $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ subsets. The same authors described that enhanced NKp46 mediated killing is associated with liver pathology. In chimpanzees, NKp46 was also up-regulated, but only in the $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$, and $CD94^{low}CD16^{high}$ subsets. Unlike humans, our chimpanzees did not develop

fibrosis in the liver after HCV infection. However, they did show signs of liver inflammation [32]. In this respect, it would be interesting to investigate intrahepatic NK cells for the expression of NKp46 and cytolytic capacity.

In chimpanzees, discordant results were observed between NCR expression and NCR mediated CD107 expression. In the $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$ and $CD94^{high/dim}CD16^{high}$ subsets high NKG2A expression was observed. However, NKG2A triggering failed to induce CD107 expression. By contrast, low NKG2A expression was detected on the surface of $CD94^{low}CD16^{high}$ cells, but the cells showed only modest NKG2A mediated CD107 expression. In contrast to earlier published data, where NKG2A was found up-regulated on NK cells from individuals with chronic HCV [12], the results presented here show increased NKG2A-induced cytotoxicity in both naïve as well as HCV infected chimpanzees. The NKG2-family is well conserved between different primate species but exhibit polymorphism that affect the binding of monoclonal antibodies [33]. The antibody that was used in the experiments described here is Z199. In humans, Z199 binds to NKG2A and NKG2E. By contrast, in rhesus macaques Z199 binds to NKG2A and NKG2C [34]. The exact binding pattern of this monoclonal antibody in chimpanzees has not been investigated. While the NKG2A/CD94 complex delivers an inhibitory signal, the NKG2C/CD94 and NKG2E/CD94 deliver activating signals upon ligand binding. In this light, the NK cells in the $CD94^{low}CD16^{high}$ subset that respond to Z199 engagement with CD107 expression may not be inhibiting NKG2A cells, but also include activating NKG2C NK cells.

In conclusion, the present study provides a new strategy that enables evaluation of different NK subsets in chimpanzees. Furthermore, it shows that spontaneous and NCR mediated cytotoxicity are effector functions of distinct NK subsets in HCV infected chimpanzees.

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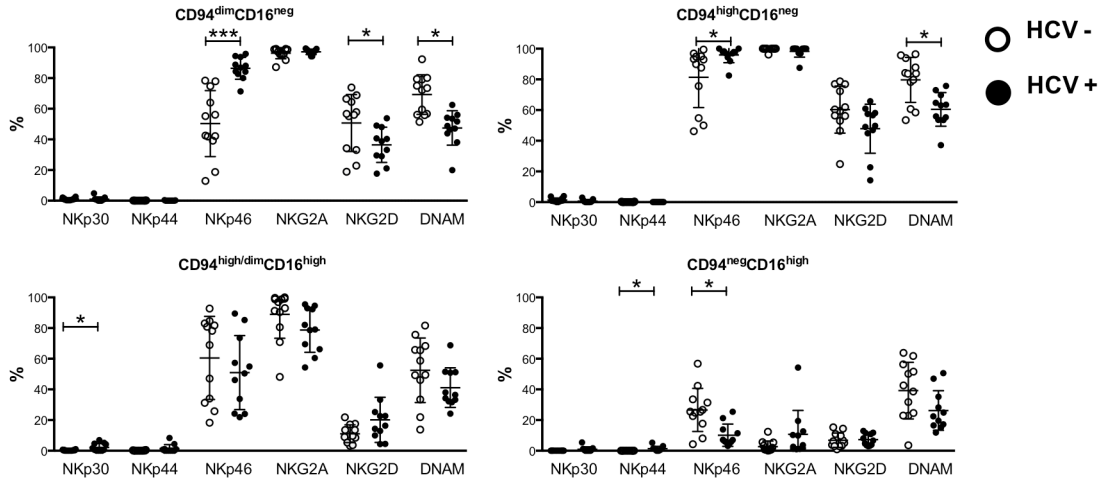
SUPPLEMENTARY FIGURE



Supplementary Figure 1; Frequency of NCR expression on NK subsets from naïve and HCV infected chimpanzees.

The frequency of NKp30, NKp44, NKp46, NKG2A, NKG2D and DNAM expressing cells was calculated as the percentage of $CD94^{dim}CD16^{neg}$ (upper left) $CD94^{high}CD16^{neg}$ (upper right) $CD94^{high/dim}CD16^{high}$ (lower left) and $CD94^{low}CD16^{high}$ (lower right) in naïve (open circles) and HCV infected chimpanzees (black dots). At the bottom of each graph the MFI is indicated in italic. The dots represent the individual chimpanzees tested. Statistical significant differences are indicated as * (p value between 0.05 and 0.001, ** (p value between 0.001 and 0.0001) and *** ($p \leq 0.0001$). For every group, the bars represent the mean value and the standard deviation. Statistical significant differences are indicated and considered significant when $p<0.05$.

4

General Discussion

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GENERAL DISCUSSION

Chimpanzees have been the most important animal model in the study of HCV [1-3]. Chimpanzees have played a key role in the discovery of the virus and characterization of HCV-induced adaptive immune responses. More recently, chimpanzees have improved our understanding of vaccine-induced protection and the consequences of viral persistence [4]. The latter were broadly discussed in the previous chapters of this thesis. The general discussion will therefore focus on the overall role of the chimpanzee as an animal model, and on new challenges in controlling the HCV epidemic.

The end of an era, changing gears.

In 2002, the Dutch Government prohibited the use of great apes for biomedical research. Since then, all chimpanzees that were housed at the BPRC have been relocated to either Zoos or a sanctuary. By then, the ethical debate on using chimpanzees had started in the USA. In 2013, it was agreed that research on NIH-owned or -supported chimpanzees was no longer allowed. Also, a number of pharmaceutical companies, including MSD, signed an agreement declaring they will no longer use chimpanzees in their research programs. Still, in the USA, a colony of 50 chimpanzees will be kept for future research. Any new research proposal involving chimpanzees, will be evaluated by 'the chimpanzee research use panel', before the NIH makes a decision about funding. Approval will only be given for exceptional research subjects. HCV is one of the viruses on the list of exceptions.

Shortly after the public debate started on the ban of the use of chimpanzees for research purposes, *in vitro* models to study HCV became sparsely available. Critics may believe this timing is not a coincidence, but many documented attempts are available showing the hurdles that were overcome before *in vitro* culture systems were a fact. Even today, it is not possible to isolate HCV from any given patient, and replicate that virus *in vitro*. Only one successful attempt has been described, JFH-1 [5]. JFH-1 was isolated from a patient with fulminant hepatitis and full length HCV RNA was transfected into Huh-7 cells. Nowadays, JFH-1 is widely used and chimeric viruses, combining JFH-1 and other HCV genotypes and subtypes, have been generated (reviewed in [6]).

The progress made in the development of these *in vitro* systems resulted in better understanding of the HCV lifecycle and virus-host

interactions. It is safe to say that these cell culture-based techniques have directly initiated the development and screening of direct-acting antiviral drugs (DAA). DAAs are compounds that specifically target nonstructural proteins of HCV and disrupt virus replication. Currently, DAAs can be divided in four classes, NS3/4A protease inhibitors, NS5A inhibitors, NS5B nucleoside and NS5B non-nucleoside inhibitors. The year 2011 is considered a turning point in the treatment of chronic HCV infection. In that year telaprevir [7] and boceprevir [8] received regulatory approval, and since then, other compounds have followed [9].

Immense improvement of HCV management options

Since the introduction of the first DAAs, new generation drugs have been developed, evaluated and approved. Unlike HIV treatment, which only controls infection and must be taken for the rest of a patient's life, HCV treatment aims at the elimination of the virus. To determine the most effective treatment approach, extensive pre-therapeutic diagnostics are required. This includes the determination of liver disease severity, HCV RNA quantification, genotype and subtype assessment. The full medical history of a patient is important as coinfection with hepatitis B virus, HIV are comorbidities that increase progression of liver disease and adaptation of treatment protocol may be required. Also other non-liver related disorders may require modification of treatment. Approximately 90% of the patients can now be successfully treated [10].

The success of the new treatment options has led to new concerns. DAA-therapies are much more expensive than IFNα/RBV-treatment and not every HCV-infected patient can be treated immediately, therefore prioritization is necessary. To determine the impact of treatment with respect to medical costs and how society benefits from quality-adjusted-life-years, everal cost-effectiveness-studies have been performed. [11-13]. The multiparametric nature of these types of analysis make it difficult to compare the results but they are used to create decision models for HCV management. In Europe, 'the EASL Recommendations on Treatment of Hepatitis' [10] describes the clinical decision-making process for optimal HCV management. In November 2015, subcutaneous injection of PegIFN-α2a, PegIFN-α2b and the orally administered Ribavirin, Sofosbuvir, Simeprevir, Daclatasvir, Ledipasvir, Paritaprevir, Ombitasvir, Ritonavir and Dasabuvir were available and combined in various regimen. The current challenge in the development of new drugs lies in the care of difficult to treat patient groups, like patients

infected with genotype 3, or more advanced fibrosis stages, renal impairment/hemodialysis, HIV coinfections, decompensated cirrhosis and post-transplant patients. Also the development of new pan-genotypic agents is of great interest.

Are DAAs the holy grail for controlling HCV epidemic?

The reverse side of recent developments, like the second generation DAAs and ethical debate on using chimpanzees, is that HCV vaccine development has nearly come to an end. The new drugs shifted a vaccine further down the list of priorities. However, when we look at the developments in the HIV-field, this may change in the near future.

Initially, antiviral therapy gave HIV infection the status of chronic disease instead of a deadly disease. Years after the introduction of HAART, it is obvious that treatment is a major step forward, but it will not put a halt to the HIV epidemic. On one hand, long-term use of antiviral drugs exerts continuous selective pressure on the virus and may eventually lead to viral resistance. On the other hand, every day 5600 people [14] are newly infected with HIV. The majority of these people live in Africa and do not have access to a proper health care system and antiviral therapy is out of reach. Therefore, immunizing high-risk individuals in endemic areas, is currently seen as the best option to stop the HIV epidemic.

Similar circumstances apply to the HCV epidemic. It is without question that DAAs are a great improvement in patient care and HCV management in the developed parts of the world but it requires a large financial investment. Cost effectiveness of treatment with the new agents depends on many factors and also involves agreement between the pharmaceutical industries, local authorities and public health care services [15]. The price tag for treatment in China, where 200 to 450 million people are infected [16] is different from Egypt, with 8-10% the highest HCV prevalence in the world. But for both governments the costs are contingencies that initially require a large investment [17]. This implies that the new generation of drugs may not be available to patients in developing countries, or in countries with relative low prevalence.

Additionally, the primary goal of treating chronic HCV infection is to eliminate the virus and to reduce the risk of development of hepatocellular carcinoma (HCC). But the association between drug-induced viral clearance HCV

disease regression is controversial. Several studies have reported that drug-induced clearance is associated with reduced liver fibrosis [18-20] and decreased incidence of HCC· [21-26]. However, others have reported on the development of HCC after viral clearance [27-32]. These studies indicate that viral clearance does not warrant prevention of disease progression. A recent meta-analysis from 25 observational studies showed a 10% reduced risk of developing HCC compared between patients with and without SVR [33], 6.7 % in patients without cirrhosis and 22 % in patients with cirrhosis. The mechanism behind progressive liver disease after therapy-induced viral clearance is currently not understood. Possibly, HCV remains present in the liver at very low levels and local replication is responsible for disease progression. This is in line with the observation of HCV-specific CD8 T cells in the liver of a successfully treated chimpanzee [34] and intrahepatic Tregs in patients 4 years after HCV clearance [35].

Another concern is the development of viral resistance. Although there are basic differences between HIV and HCV, similar mechanisms have been reported to contribute to viral resistance as a result of antiviral therapy. Control of virus replication can be achieved by interfering with the function of the accessory, nonstructural proteins. This selective pressure may cause adaptation of the virus resulting in the formation of escape variants. Viral resistance has already been reported to protease inhibitors [36-38]. On the positive side, mutations induced by selective pressure, can lead to a reduction in viral fitness. It was demonstrated that immune pressure induced changes of non-structural regions can be lethal to the virus [39] and potentially limit viral persistence. Still, the potential development of antiviral resistance is of great concern that may interfere with controlling the HCV epidemic.

Back to the drawing board

An affordable vaccine that is able to prevent primary infection or reinfection of people in high-risk groups, or cure chronic HCV infection would therefore be of benefit.

Reinfection studies in humans and chimpanzees have shown that previously cleared HCV infection does not confer life long immunity [40-43]. It can however reduce the development of persistent HCV infection. Based on this, several prophylactic vaccine efficacy experiments were performed in chimpanzees [44-58]

Relevant information regarding vaccine components, strategy, adjuvants, genotype of the vaccine-components and the challenge virus, and

the challenge outcome are summarized in Table 1 and reviewed by Verstrepen et al [4]. In summary, these studies have shown that HCV vaccine efficacy is a case of trial and error. Protection from infection is rare [44, 55] but vaccine-induced responses may protect from viral persistence [45-56, 58, 59]. More importantly, HCV vaccine experiments have revealed new insights in potential mechanisms that are involved in vaccine-induced protection from chronic HCV infection. For instance, it was found that recombinant E1/E2 envelope proteins can elicit antibody responses that may protect from heterologous infection [44]. This is not surprising, as E2 has been described to contain several neutralizing epitopes that potentially block binding to CD81, SRB1 and coreceptors [60]. Because these HCV-envelope protein vaccines were based on the E1/E2 heterodimer and the role of the individual glycoproteins could not be determined. Only recently, E1 and E2ΔHVR-1gp (E2 lacking the HVR-1) were evaluated separately [47]. In two animals immunized with gpE1, HCV neutralizing antibodies were induced. Both animals were able to resolve HCV infection shortly after a heterologous HCV-1b challenge. In contrast, the two E2ΔHVR-1 immunized animals showed no neutralizing capacity and, despite the presence of E2 specific cellular responses, both animals became chronically infected. This study showed for the first time that E1 neutralization can be achieved by vaccination. This may imply that blocking E1 epitopes interferes with proper gpE1/E2 folding and sheds new light on the role of E1 as an immunogen in HCV vaccines.

Many HCV vaccine approaches that aim to induce T-cell responses, like virus-like particles and multicomponent prime-boost modalities, have been evaluated (Table 1). Overall broad and strong vaccine induced T-cell responses were associated with better perspectives but did not warrant protection from infection. In our DNA/MVA experiment, four animals were immunized with HCVcore-E1-E2 and HCV-NS3 plasmids and MVA expressing HCVcore-E1-E2 and HCV-NS3. Strong and broad T- and B-cell responses were reported [53]. However, despite strong humoral responses, no virus neutralizing capacity was found and after challenge with HCV-1b, all four animals showed acute viremia. Only one animal was able to control virus replication to undetectable levels. The other three animals became chronically infected. The vaccine induced vigorous T-cell responses as reflected by strong proliferation and HCV specific IFNγ, IL-2 and IL-4 responses. Retrospectively, vaccine induced T-cell responses were analyzed in more detail. It was found that, although the vaccine elicited NS3 specific cytokine producing CD4 and CD8 T-cells in all four vaccinees, only in the chimpanzee

that cleared HCV infection, CD8 T-cells were found to have cytolytic capacity [61]. Interestingly, the animals that became chronically infected had higher mRNA expression levels of exhaustion markers PD-1, CTLA-4 and IDO in the liver, suggesting the induction of T-cells with regulatory functions that might have prevented formation of a cytotoxic T-cell response [53]

The induction of functional cytolytic T-cells by vaccines did not warrant protection from infection as shown by Folgori [56] and chapter 2.1 of this thesis. On the positive side, we have shown that cytolytic T-cells may protect from viral persistence (this thesis chapter 2.2). This is an important finding as the induction of killing competent T-cells was only achieved in the animal with the appropriate MHC class I molecule to present the viral peptide to the T-cell. This finding implies that peptides that are selected after screening of the HCV genome for conserved CTL-epitopes, and have a high binding capacity to MHC class I molecules, may be used as vaccine immunogens. To be able to protect at population levels, rather than individual level, several peptides covering different HCV genotypes and class I molecules, should be included in a vaccine. Similar synthetic peptide approaches are currently under investigation for malaria [62], HIV [63] and human papillomavirus [64].

Hypothetically, this result may also be an opening towards a therapeutic HCV vaccine-approach, similar to strategies that are currently investigated in end stage melanoma patients [65]. Using the same strategy in HCV infected patients would imply the following: First, the patient is typed for its MHC class I and the presence and variability of CTL and neutralizing antibody epitopes. Next, functional CTL and plasma cells are isolated from the patient. After *in vitro* expansion, high numbers of autologous CTL are infused into the patient to kill infected hepatocytes. At the same time, a high dose of autologous neutralizing antibodies is produced and the plasma cells are administered to prevent infection of new cells.

In summary, these experiments have provided evidence for the central role of neutralizing antibodies in obtaining protection from HCV infection. On the other hand, most vaccine candidates that induce cellular immune responses do not protect from infection but lead to reduced viremia in the acute phase of the infection and reduce the risk of viral persistence. The current challenge is to translate this newly acquired knowledge into an efficient prophylactic or therapeutic HCV vaccine with improved efficacy.

Surrogate model, the next best thing?

Due to further restrictions on the use of chimpanzees for biomedical research, future evaluation of new vaccine candidates in these animals will be limited. In recent years, strong effort was made in the development of a small, fully immunocompetent, animal model that supports HCV replication. So far a wide range of rodent models is available [6, 66]. All these models have great value in specific HCV research fields, but lack at least one of the important features that are required to study HCV vaccine efficacy.

Like SIV is used to study HIV infection in humans [67], HCV may also benefit from a surrogate model. Although the experimental infection of bank voles with a newly discovered hepacivirus is promising [68], a rodent model may have its limitations, partly because innate immunity is differently regulated in rodents compared to humans [69]. GBV-B in common marmosets is a promising candidate in non-human primates. Worldwide, several experiments have been performed to characterize the infection of GBV-B in marmosets. With the report on persistent GBV-B infection in the presence of immune mediated liver pathogenesis and fibrosis [70, 71], GBV-B may have the potential to become the new non-human primate model to study HCV infection. In addition, common marmosets are already broadly used in biomedical research in the fields of autoimmune- and infectious diseases [72, 73]. This implies that both innate as well as the adaptive immune system of these new world monkeys resembles the human system. With common mamosets being scientifically accepted models for various types of disorders, more specific diagnostic tools become available to investigate immune responses in these animals [73]. A non-human primate model to study HCV infection would be of great value, in addition to the rodent models that are currently available.

A recent report that primary hepatocytes from rhesus macaques are permissive for HCV replication, and that particle production can be enhanced by suppression of antiviral signaling pathways, is an exciting breakthrough [74]. This development indicates that rodent models alone, do not suffice when aiming at the dissection of the complex interplay between the immune system, HCV pathogenesis, lentiviral coinfection and vaccine development.

Misconceptions in HCV research in chimpanzees.

Investigating human diseases in an animal model, especially non-human primates and chimpanzees, often leads to debate on ethical considerations versus translational value. Without a doubt, the preferable species to study HCV infection is humans, however it is not possible to control conditions, like time point, genotype and dose of infection. Only a well-designed and properly conducted animal-experiment allows standardization of these variables. This does however imply intrinsic differences between the natural host and the experimental model. The role of the chimpanzee, in dissecting the adaptive immune responses during acute and chronic HCV infection, is indisputable. The value of chimpanzees in investigating other immune mechanisms, like immune-mediated pathology during is HCV infection, is however less well-recognized. The final paragraphs of this thesis are used to discuss some of the misconceptions that are commonly held about HCV research in chimpanzees.

An often-used point of criticism is that chimpanzees do not develop hepatic fibrosis and are therefore not good models to study immune-mediated pathology. However, it is well recognized that some humans do not progress to fibrosis, while others rapidly develop significant fibrosis [75]. In fact, male sex, duration of infection, acquisition of infection at >40 years of age, long term excessive alcohol consumption, long-term immunosuppression in case of HIV coinfection or organ transplantation and coinfection with hepatitis B virus are typical determinants for HCV-induced fibrosis. Some of these factors do not apply in the controlled laboratory settings that are associated with chimpanzee research. Typically, healthy, young-adult animals are selected for this type of research and maybe even more important, alcohol and other toxic substances that are either cleared by or metabolized in the liver, are not part of their daily diet. Therefore, it is tempting to speculate that chimpanzees resemble human non-progressors.

No doubt, innate immune responses are differently regulated between chimpanzees and humans. Qualitative analysis of LPS induced immune responses have shown many similarities in regulatory pathways but also differences between humans, rhesus macaques and chimpanzees [76].
Microbial translocation is not restricted to the gut, but also takes place in other mucosal surfaces, like the skin, lungs, eyes, nose, mouth, throat and parts of the reproductive organs. Obviously, chimpanzees have lower hygiene standards compared to humans, and It is therefore expected that the bacterial

burden in peripheral blood is higher and sCD14 levels are more up-regulated in these animals. However, our experiments have shown lower levels of sCD14 in chimpanzees. In addition, it was observed that in chimpanzees, monocytes express lower levels, or even no CD14, on their surface. Although highly speculative, this may be a direct result of the divergent evolution of human and chimpanzee species. In line with this finding is the lower expression of NKp30 and CD56 on the surface of chimpanzee NK cells. Our data has shown that chimpanzees do respond to LPS. Similar to HIV infected humans, chimpanzees show signs of gut perturbation with up-regulated levels of sCD14, LPB and IL-6. The overall response observed in chimpanzees was lower compared to humans and it would be interesting to investigate if the human system is overreacting or if there is dampening of the immune system in chimpanzees [77].

It was described that chimpanzees do not respond to IFNα-based therapy [78]. This was explained by stronger up-regulation of intrinsic IFN pathways by HCV infection in chimpanzees compared to humans. In patients chronically infected with HCV, low baseline IP-10 levels are predictive for successful treatment-induced clearance [79] Indeed, we observed much higher levels of IP-10 in some of the chronically infected chimpanzees. In approximately 50% of the animals, up-regulation of IP-10 levels was comparable to humans, suggesting that chimpanzees are more similar in this respect than earlier concluded. Lanford et. at. [78] concluded that chimpanzees overall, resemble human non-responders to IFN-based antiviral treatment. However, they analyzed only a limited number of chimpanzees and at least two of these animals had a high virus load. Based on our finding that virus load correlated with peripheral IP-10 levels, it is to be expected that IP-10 levels in these animals are high. It is therefore tempting to speculate that chimpanzees with a high HCV load, and subsequent high baseline IP-10 levels, are indeed equivalent to human non-responders to IFN-based therapy. However, this implies that animals with lower IP-10 levels may respond to IFN-based therapy. Unfortunately, this was never tested in chimpanzees.

Another often suggested difference is that spontaneous clearance is more often observed in chimpanzees compared to humans and therefore chimpanzees are not comparable to humans. For decades it was estimated that approximately 80% of the people who contract HCV, become chronically infected. However, improved screening programs show that this is overestimated. Recently WHO has adjusted the percentage of HCV-

persistence to 55 to 85%. This is in line with the finding of HCV specific cellular immune responses in seronegative individuals, implying loss of HCV-specific antibodies after viral clearance can occur in humans [80-83]

On the other hand, there may be a good explanation for the firm believe that chimpanzees clear HCV infection more often compared to humans. Humans and chimpanzees share a common ancestor who lived approximately 5 million years ago. After the split of the hominoids, both species evolved separate from one another. This implies that both species encountered different –lethal- pathogens. One of the theories is based on evidence for a selective sweep in chimpanzees, which led to a reduction of the MHC class I repertoire [84]. The allotypes that were preserved after the selective sweep are enriched for binding peptides that are commonly presented by HLA-B27 and HLA-B57, the alleles that are associated with protective HCV specific immune responses and HCV clearance [84].

Closing remarks

Treatment of chronic HCV has improved after the regulatory approval of DAAs. However, high costs, ongoing disease progression and viral resistance are of concern. A prophylactic or therapeutic vaccine is therefore a good alternative. Over the years, two types of prophylactic vaccines were evaluated in chimpanzees. The envelope-based vaccines that induce neutralizing antibodies and vaccines that induce HCV specific T-cells are predominantly based on more conserved non-structural proteins.
The results discussed in this thesis have shown that strong vaccine-induced immune responses do not protect from HCV infection, but may protect from viral persistence.

- We have shown that immunization with E1 can induce neutralizing antibodies. This was the first time that E1 alone was used as an vaccine and both animals generated E1-neutralizing antibodies. More importantly, both animals cleared the infection rapidly after exposure. These results may have important implications for vaccine development.

- In another vaccine study we found that protection from chronic infection was associated with the induction of killing competent CD8 T-cells. Additionally we showed an association between vaccine-induced cytolytic T-cells and the genetic background of the animal. These

results emphasize the importance of the MHC system in the induction of fully functional effector T-cells.

- In order for a prophylactic vaccine to be effective, it should be administered before the subject is exposed to the pathogen. Given the majority of the new infections in the Western World are identified in intravenous drug users, one can imagine that this and other high risk groups, could benefit from this type of vaccine. On the other hand, this thesis also provides data that may help to design a successful therapeutic approach. A therapeutic vaccine should meet other requirements compared to a prophylactic vaccine. Many mechanisms have been associated with immune dysfunction and HCV persistence. We have shown that low levels of expression of PD-1 in hepatic tissue was associated with HCV clearance [53]. This suggests that a combination of restoration of the T-cells with monoclonal antibodies and a vaccine that boosts the CD4 and CD8 T-cell responses, may be an effective therapeutic approach. Therapeutic vaccines that are currently in clinical development [85], in combination with biological compounds like anti-PD-1 or anti-CTLA-4 or one single DAAs may be as effective as treatment current combinatorial DAA treatment, but less sensitive for antiviral resistance and less expensive.

The final chapters of this thesis were committed to the consequences of HCV infection, and the value of the chimpanzee to investigate this.

- Genetic diversity throughout different species and within one species is a characteristic of evolution. We have shown that the polymorphism near the IL-28B gene, which is associated with the outcome of human HCV infection, is not present in chimpanzees. We tested two SNPs and chimpanzees are homozygous for both alleles. One SNP represents the ‘risk allele’ while the other SNP represents the ‘good allele’. These data suggest that the polymorphism in humans was not driven by HCV as the selective marker, as one would expect that chimpanzees would either have ‘risk alleles’ ‘good allele’. This is a good example of how human medicine should benefit from translational research in animal models.

- It is firmly believed that chimpanzees do not respond to IFN-based antiviral treatment, but this was based on one experiment in three

chimpanzees. According to literature, at least two of them have a high virus load. In this thesis we provide data that chimpanzees with a high virus load resemble human non-responders. This may indicate that chimpanzees with a low virus load may benefit from IFN-based antiviral treatment. This was however never tested.

- The translocation of bacterial products and consequent chronic systemic immune activation plays an important role during HIV associated disease progression [86]. Increased microbial translocation has also been associated to HCV infection and liver fibrosis [87]. In this thesis, we provide data that sCD14 is up-regulated in chimpanzees after HCV infection. Chimpanzees do typically not develop liver fibrosis as a result of HCV infection, and no evidence was found for mucosal damage of the small intestines in these animals. This suggests that the elevated sCD14 observed in HCV infected humans is not mediated by portal hypertension caused by fibrosis alone, as currently assumed.

- Because of differential CD56 expression between human- and chimpanzee NK cells, it was not possible to divide the chimpanzee NK population into subsets; chimpanzee NK cells cannot be subdivided into $CD56^{dim}CD16^{pos}$ and $CD56^{bright}$ cells. In this thesis we describe a new strategy to identify 4 functionally distinct NK cells based on the expression of CD94 and CD16; $CD94^{dim}CD16^{neg}$, $CD94^{high}CD16^{neg}$, $CD94^{high/dim}CD16^{high}$ and $CD94^{low}CD16^{high}$ and enables the functional analysis of these cells.

Table 1; Summary of vaccine experiments in chimpanzees.

VACCINE				CHALLENGE		OUTCOME					
COMPONENTS	ADJUVANT	ROUTE (prime-boost)	GT	STRAIN	DOSE (CID50)	# STERILE	# CHRONIC	# RESOLVED	#TOTAL	%CHRONIC	ref
Recombinant protein											
E1E2	MF59/MF57	i.m.	1a	HCV-1	10	5		2	7	0	44
E1E2			1a	HCV-1			2	12	14	14	45
E1	ALUM	i.m.	1b	HCV 1b J4	100			2	2	0	47
E2deltaHVR-1	ALUM	i.m.	1b	HCV 1b J4	100		2		2	0	47
Recombinant protein-peptides											
E1, E2, HVRpeptides	(in)complete Freund's	s.c.	4	HCV#6	10			1	1	0	46
DNA											
E2	none		1a	1a	100			2	2	0	48
Virus like particle											
Core, E1, E2	AS01B	i.m.	1b	HCV CG 1b	100			4	4	0	49
DNA protein											
Core, E1, E2 and NS3	ALUM	i.m./i.d.-i.m.	1a / 1b	HCV 1b J4	25		1	1	2	50	50
DNA-peptide protein											
E1/E2 +HVR peptides	ALUM/RIBI	i.m.	1a	H7	100		1		1	100	51
DNA prime- vaccinia boost											
NS3, NS5A, NS5B	CpG, rVV B7.1; ICAM-1; LFA-3	i.m./s.c.	1a	H77	100		1		1	100	52
DNA prime - MVA boost											
Core, E1, E2 and NS3	none	i.m./i.d. - i.m/i.d.	1b	HCV 1b J4	25		3	1	4	75	53
Replicating rVV											
Core, E1, E2, p7, NS2 and NS3	none	i.d.	1b	HCV 1b BK	2.5 and 24			4	4	0	54
DNA prime - Adeno boost											
Core, E1, E2, NS3-NS5	With/without IL-12	i.m.-i.m.	1b	HCV 1b BK	100	1	4	1	6	67	55
NS3 - NS5B	none	i.m./i.m.	1b	H77	100		1	4	5	20	56
NS3, NS4, NS5A, NS5B	Liposomes/pIL12	i.v.-i.v.	1b	H77	100		2	2	4	50	57/58

i.m.; intramuscular, i.v.; intravenous, rVV; recombinant vaccinia virus, rMVA; recombinant modified vaccinia virus, s.c.; subcutaneously.

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5

Summary/Samenvatting.

SUMMARY

Approximately 1 to 2 % of the world population is chronically infected with hepatitis C virus (HCV). Of these people, 15 to 30 percent will develop liver cirrhosis or liver cancer within 20 years after infection. HCV is therefore not just a social problem but also a large burden for the health care system.

Only one in five individuals is able to spontaneously clear the virus. Since acute infection generally does not cause clinical signs, people are not aware of the fact that they are infected. As a result, the complicated interactions between the virus and the host early after infection are difficult to study in humans. Moreover, reliable baseline sampling is complicated to organize. An animal model circumvents these challenges, and enables experimental infection under controlled conditions.

In the early 80s of the last century, HCV was identified as the causative agent of non-A-non-B hepatitis. Chimpanzees have played a crucial role in the discovery of HCV, identification of the modes of transmission of the virus and characterization of the course of HCV infection. More recently these animals were used to evaluate vaccine candidates. Growing ethical awareness has led to a ban on the use of chimpanzees for biomedical research in Europe since 2003. With the near disappearance of chimpanzees as an animal model on one side, and the approval of direct acting antiviral drugs by regulatory agencies on the other, this thesis is positioned in an era of changing perspectives on HCV research. It summarizes the key role of chimpanzees and elaborates on potential mechanisms behind successful prophylactic vaccine candidates. In addition, consequences of chronic HCV are discussed as well as the translation to the human situation.

Many studies in chimpanzees have shown that clearance early after HCV-infection is associated with relatively strong CD4 and CD8 T-cell responses, but there is also a role for neutralizing antibodies. In addition, innate immune responses have been described to play a role in controlling the infection. The similarities and dissimilarities between humans and chimpanzees are discussed in **chapter 1**. The aim of the research described in this thesis was to investigate HCV-related immune responses in chimpanzees as a model for infection in humans. In the first part, vaccine-induced immune responses, and their potential contribution to protection from chronic HCV-infection were studied. In the second part, the consequences of

chronic HCV infection on the immune system are discussed. The majority of the experimental work described in this thesis was performed in chimpanzees and, when relevant, compared to humans.

If one thing, HCV vaccine studies have shown that efficacy of the vaccine is very much a case of trial and error. **Chapter 2.1** describes the evaluation of a so-called prime-boost vaccine efficacy study. In this study, four chimpanzees first received a HCV-DNA prime followed by an HCV-MVA boost. The vaccine elicited strong immune responses in all four animals. However, after experimental infection, in three animals the virus persisted, whereas one animal cleared the infection. Analysis of the CD4 and CD8 T-cell responses by intracellular cytokine staining was not conclusive in explaining why. To examine this in more detail, a FACS-based cytotoxicity-assay was developed. With this assay, we showed that the vaccine elicited killing-competent CD8 T-cells in the animal that cleared the infection, whereas these specific vaccine-induced cells were not present in the animals that became chronically infected. This is discussed in detail in **chapter 2.2**.

In **chapter 2.3**, the protective role of vaccine-induced E1-neutralizing antibodies are discussed. While the HCV-E2 protein is known for its epitopes that bind to neutralizing antibodies, HCV persisted in two E2-immunized animals after experimental HCV infection. By contrast, two other animals that received a recombinant E1-vaccine, were able to clear the virus shortly after infection. Using the HCV pseudoparticle neutralization assay, it was found that serum from the E1-immunized animals could protect *in vitro*-cultured cells from infection with a heterologous HCV 1b virus. Serum from the two E2-immunized animals did not show this protective capacity.

Chapter 3 of this thesis focuses on the consequences of HCV infection. We investigated serum IP-10 levels in chimpanzees and compared these with the levels in human patients. IP-10 is a protein that is part of the innate immune system and that was found to be strongly up-regulated in patients that are difficult to treat. In **chapter 3.1**, the up-regulated levels of IP-10 in chimpanzees are discussed. These data suggest that, unlike the dogma, some chimpanzees may respond to IFN treatment. We were unable to test this hypothesis at the BPRC.

In HIV patients, it was found that microbial translocation, the transfer of microbial residues from the gut lumen into the peripheral blood, is increased.

This was also found in HIV patients that were co-infected with HCV, but in patients that were infected with HCV alone, the results were inconclusive. However, it was postulated that liver fibrosis, as a consequence of HCV infection, would affect the clearance of bacterial products from the blood explaining the higher levels in humans with fibrosis. To examine this, we analyzed serum soluble CD14 levels in human patients with HCV-induced fibrosis, and compared the results with chimpanzees in whom typically no fibrosis is seen. We found up-regulated sCD14 levels in both humans and chimpanzees infected with HCV, suggesting that up-regulation of sCD14 can also occur in the absence of liver fibrosis. In addition, we showed that increased sCD14 levels during HCV infection are not a result of increased gut-perturbation. By contrast, in a group of HIV/SIV infected chimpanzees sCD14 up-regulation was accompanied by more enterocyte death and thus leakage of bacteria into the blood. These data show that microbial translocation does occur in chimpanzees, but not as a result of HCV infection. We discuss these findings in **chapter 3.2**.

In the next chapter, we discuss yet another aspect of the innate immune system, the NK cells. In humans and rodents, functionally distinct NK subsets can be identified based on the expression of CD16 and CD56. This is not possible in chimpanzees. In **chapter 3.3** a new method is described to distinguish between different NK subsets in chimpanzees. Next, we used this method to study the impact of HCV infection on NK subsets in chimpanzees. We observed spontaneous and NCR mediated killing were functional characteristics of different subsets in HCV infected chimpanzees.

In the final chapter, **chapter 4**, an overview is presented of all prophylactic vaccine experiments that were performed in chimpanzees worldwide. In addition, a picture is painted of future HCV research without chimpanzees, and the pro and cons of this new era are discussed.

SAMENVATTING

Naar schatting 1 tot 2 procent van de wereldbevolking is chronisch geinfecteerd met het hepatitis C virus (HCV). Hiervan zal 15 tot 30 procent leverfalen ontwikkelen. HCV is dan ook niet alleen een maatschappelijk probleem maar legt het ook een enorme druk op het gezondheidssysteem.

Slechts een op de vijf mensen beschikt over de juiste tools om een besmetting met HCV adequaat te lijf te gaan. Dit betekent dat het virus in de rest van de patiënten een manier vindt om blijvend aanwezig te zijn, met een chronische infectie als gevolg. Doordat er kort na infectie nauwelijks sprake is van klinische symptomen, zijn mensen zich meestal niet bewust van HCV besmetting en worden dus niet door een arts gezien. Hierdoor is het bijna onmogelijk om een goed beeld te krijgen van hoe het virus en het immuunsysteem op elkaar reageren in deze vroege fase. Daarnaast is het ingewikkeld om bijvoorbeeld seriële bloedmonsters te verzamelen van dezelfde personen, inclusief van tijdstippen vóór de infectie heeft plaats gevonden. Een diermodel is de enige mogelijkheid om dit soort problemen te omzeilen en een infectie uit te voeren onder gecontroleerde omstandigheden. Tot op heden is de chimpansee de enige diersoort waarbij dit mogelijk is.

In de vroege jaren 80 van de vorige eeuw werd de voornaamste veroorzaker gevonden van de zogenaamde non-A-non-B hepatitis, HCV. Chimpansees hebben een cruciale rol gespeeld bij deze ontdekking en later ook in de onderzoeken naar hoe het virus wordt overgedragen en hoe de infectie zich verder ontwikkelt. Daarnaast hebben chimpansees bijgedragen aan de ontwikkeling en evaluatie van potentiele vaccins. Meer kennis over chimpansees en hun zelfbewustzijn heeft geleid tot een ethische discussie over het gebruik van chimpansees in biomedische onderzoek. Mede hierdoor is het sinds 2003, binnen Europa, niet meer toegestaan om mensapen te gebruiken voor deze doeleinden. Met het verdwijnen van het enige gevalideerde proefdiermodel aan de ene kant, en doordat de autoriteiten goedkeuring hebben gegeven voor het gebruik van nieuwe antivirale middelen om HCV infectie te behandelen aan de andere kant, is het HCV onderzoek in een nieuw tijdperk beland. Dit proefschrift beschrijft de belangrijke rol die chimpansees hebben gespeeld in het HCV onderzoek.

Door de jaren heen hebben studies in chimpansees laten zien dat spontane klaring van HCV geassocieerd is met relatief sterke CD4 en CD8 T-

cel responsen. Ook is er een duidelijk een rol voor neutraliserende antilichamen en zelfs het aangeboren immuunsysteem. De overeenkomsten en verschillen tussen mensen en chimpansees tijdens HCV infectie worden uitvoerig beschreven in de algemene inleiding in **hoofdstuk 1**. Het wetenschappelijk deel van dit proefschrift was gericht op het kaart brengen van de immuun-responsen die chimpansees ontwikkelde tegen HCV, om zo de infectie in mensen beter te begrijpen. In het eerste deel worden met name vaccins besproken. Er worden immuun-mechanismen uitgelicht die mogelijke ten grondslag hebben gelegen aan de beschermende capaciteit van die vaccins. In het tweede deel is gekeken naar de mogelijke gevolgen van chronische HCV infectie op het immuunsysteem. Het overgrote deel van het werk dat wordt beschreven in dit proefschrift gaat over chimpansees en daar waar mogelijk worden de resultaten vergeleken met mensen.

Wanneer het over HCV vaccins gaat, is er één ding duidelijk. Aan de hand van eerdere resultaten is niet te voorspellen hoe beschermend het effect van een vaccin is. In **hoofdstuk 2.1** wordt een vaccinstudie in chimpansees beschreven. In deze studie werd een zogenaamde prime-boost strategie getest. Eerst wordt het immuunsysteem van de chimpansees ‘geprimed’ met een HCV-DNA vaccin om daarna ‘geboost’ met een ander vaccin, HCV-MVA. De vier dieren in de studie reageerde erg verschillend op de experimentele infectie die volgde en het vaccin bleek slechts één van de vier dieren te beschermen tegen chronische infectie. Omdat in eerste instantie niet duidelijk was waarom deze vaccin-strategie zo een verschillende uitwerking had in de individuele dieren, zijn we de CD4 en CD8 T-cel responsen in meer detail gaan onderzoeken. Omdat intracellulaire cytokine expressie geen verklaring bieden, daarom hebben we een nieuwe techniek ontwikkeld met behulp van de FACS. Met deze zogenaamde cytotoxiciteits-assay hebben we uiteindelijk kunnen aantonen dat de chimpansee die het virus geklaard heeft, de juiste genetische achtergrond had om precies de juiste cellen op te leiden om het virus te kunnen aanvallen. De uitkomst van dit onderzoek laat zien dat er mogelijk gedacht moet worden aan maatwerk-vaccins in plaats één vaccin voor iedereen, dit wordt bediscussieerd in **hoofdstuk 2.2**.

In **hoofdstuk 2.3** wordt dieper ingegaan op de beschermende rol van E1-neutraliserende antilichamen. HCV heeft twee envelop eiwitten aan de buitenkant van het virus deeltje, E1 en E2. Vanuit de literatuur is bekend dat E2 een aantal epitopen bevat voor neutraliserende antilichamen. In E1 zijn die in mindere mate aanwezig. Omdat de meeste vaccin-experimenten

gebaseerd zijn op een combinatie van E1 en E2 was er geen kennis over de individuele eiwitten. Wij hebben twee chimpansees gevaccineerd met E1, en twee met E2. In de twee E2 gevaccineerde dieren konden we echter geen neutraliserende antilichamen aantonen terwijl we die wel vonden in de E1 gevaccineerde dieren. Dit was een zeer opmerkelijke bevinding omdat de twee dieren met die antilichamen ook nog eens in staat bleken te zijn het virus te klaren. Hiermee hebben we aangetoond dat E1 een belangrijke rol kan spelen in de bescherming tegen HCV.

In hoofdstuk 3 focussen we ons op de consequenties van chronische HCV infectie. In mensen is gevonden dat het eiwit IP-10 een belangrijke voorspellende waarde heeft voor de behandeling van HCV met algemene antivirale middelen. Een lage IP-10 concentratie in bloed geeft aan dat de kans van slagen groter is dan een hoge IP-10 concentratie. Wij hebben gekeken naar de hoeveelheid IP-10 in het bloed van HCV geïnfecteerde chimpansees en hebben deze waarden vergeleken met mensen. In **hoofdstuk 3.1** laten we zien dat de concentratie in chimpansees erg variabel is. In sommige dieren is het zelfs veel hoger dan wat in mensen gevonden is maar in ongeveer de helft is het vergelijkbaar met de concentratie die gevonden wordt in mensen die goed te behandelen zijn met IFNα/ribaverine. In tegenstelling tot het beeld dat bestaat, zou dit kunnen betekenen dat sommige chimpansees met een lage IP-10 waarde, ook succesvol met IFNα/ribaverine behandeld zouden kunnen worden. Echter dit idee hebben we niet daadwerkelijk kunnen testen in chimpansees.

In mensen die geïnfecteerd zijn met HIV vind het fenomeen microbiële translocatie plaats. Dit wil zeggen dat er kleine stukje van bacteriën die aanwezig zijn in de darmen, door de darmwand lekken en in het onderliggende weefsel terecht komen. Dit gebeurt bij iedereen, en is zelfs een voorwaarde voor een goedwerkend immuunsysteem. In mensen met AIDS gebeurt het echter iets teveel waardoor er problemen kunnen optreden. Ook in patiënten die zowel HIV als HCV geïnfecteerd zijn is deze verhoogde translocatie aangetoond. In mensen met alleen HCV zijn de resultaten van verschillende onderzoekers niet in overeenstemming met elkaar. Het idee bestaat echter dat de verhoging van de bacteriële producten in de lever niet veroorzaakt wordt door verhoogde translocatie maar dat fibrose zorgt voor een verminderde werking van de lever waardoor er minder gezuiverd wordt. Om deze theorie te testen hebben we de sCD14 concentratie in humane patiënten met lever-fibrose, vergeleken met de sCD14 concentratie in

chimpansees. In chimpansees leidt HCV infectie namelijk niet tot lever-fibrose waardoor dit een goed model is om dit te testen. Wij hebben gevonden dat sCD14 verhoogd was in zowel mensen als chimpansees die geïnfecteerd zijn met HCV. Dit geeft aan dat verhoging ook plaats kan vinden in de afwezigheid van lever fibrose. Daarnaast hebben we geen aanwijzing gevonden voor een beschadiging van de darmwand in HCV geïnfecteerde chimpansees, wat aangeeft dat HCV geen extra lekkage veroorzaakt. Om er zeker van te zijn dat microbiële translocatie wel plaats kan vinden in chimpansees hebben we ook het bloed van een aantal HIV/SIV geïnfecteerde dieren getest. Hieruit bleek dat dit fenomeen inderdaad plaats kan vinden in chimpansees maar niet als gevolg van HCV infectie. Deze resultaten worden uitgebreid bediscussieerd in **hoofdstuk 3.2**.

In **hoofdstuk 3.3** gaan we dieper in op een ander onderdeel van het aangeboren immuunsysteem en onderzochten we de NK cellen. In mensen en knaagdieren worden verschillende subsets van deze cellen beschreven op basis van expressie van CD16 en CD56. In chimpansees is deze methode echter niet mogelijk. In dit hoofdstuk beschrijven we een alternatieve strategie om de NK populatie onder te verdelen in verschillende subsets. We gebruikten hiervoor de oppervlaktemarkers CD94 en CD16. Op basis van de expressie van deze twee moleculen hebben we vier functioneel verschillende NK subsets kunnen onderscheiden. Deze vier hebben we uitvoerig bestudeerd wat betreft uiterlijk en eigenschappen. Daarna hebben we onderzocht wat de invloed is van HCV infectie op deze cellen in chimpansees. We hebben gevonden dat spontane cytotoxiciteit en zogenaamde NCR-gemedieerde cytotoxiciteit, eigenschappen zijn van verschillende NK subsets .

Tenslotte wordt in **hoofdstuk 4**, een overzicht gegeven van alle profylactische HCV vaccin studies die wereldwijd zijn uitgevoerd in chimpansees en wordt een beeld geschetst van een HCV onderzoeksveld zonder chimpansees, en de mogelijke positieve en negatieve gevolgen hiervan.

6

Appendices

ABBREVIATIONS

aa	amino acids
ADCC	antibody dependent cellular cytotoxicity
AF-700	alexa fluor 700;
AmCyan	anemonia majano cyan
APC	allophycocyanin
APC	antigen presenting cell
APC-H7	phycoerythrin-cyanine7
CCR7	chemokine (C-C motif) receptor 7
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic T-cell
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DNAM	DNAX Accessory Molecule-1, CD226
E1	envelope protein 1
E2	envelope protein 2
ECD	phycoerythrin-Texas Red
ELISPOT	enzyme-linked immunospot
ELISA	enzyme-Linked Immuno Sorbent Assay.
FACS	fluorescence-activated cell sorting
FitC	fluorescein isothiocyanate
HCV	hepatits C virus
HCVpp	HCV pseudo particles
HIV	Human Immunodeficiency Virus
ICS	intracellular cytokine staining
IDO	indoleamine 2,3 dioxygenase
IFN-α	interferon alpha
I-FABP	Intestinal fatty acid binding protein
IFN-γ	interferon gamma
IL-2	interleukin 2
IL-4	interleukin 4
IL-6	interleukin 6
IP-10	killer-cell Ig-like receptor
KIR	interferon gamma-induced protein 10
LBP	LPS binding protein
LPS	Lipopolysaccharide
MHC	major histocompatibility complex
MoAb	monoclonal antibody
MVA	Modified Vaccinia Ankara
Nab	neutralizing antibody
NCR	natural cytotoxicity receptor
NK	natural killer
NS3	non structural protein 3
PBMC	peripheral blood mononuclear cells

PCR	polymerase chain reaction
PD-1	programmed death receptor 1
PE	phycoerythrin
PE-Cy7	phycoerythrin-cyanine7
PerCP	peridinin-chlorophyll-protein complex conjugate
RT-PCR	Real-Time PCR

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Een proefschrift schrijven op mijn ouwe dag bleek een eitje, het dankwoord op papier zetten daarentegen, is een heel ander verhaal.

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CURRICULUM VITAE

Babs Verstrepen was born on March 17, 1971 in Oosterhout, The Netherlands. After graduating secondary school at the Willem van Duivenvoorde College in Oosterhout, she went to the Spectrum College in Breda to study Chemistry. She proceeded her education at the Higher Laboratory Education (HLO) in Utrecht. Babs performed her internship at the Faculty of Veterinary Medicine at the Utrecht University. Under the supervision of prof. dr. Cornelissen and dr. H Schallig she worked on the, isolation and expression of the 15 kDa excretion protein of the pathogenic nematode Heamonchus contortus. She obtained her degree in 1995 and started working at in the group of prof A.W. Cornelissen on *in vitro* induced resistance of malaria parasites against the nucleoside analogue (S)-HPMPA.
In 1996 Babs started working as a research technician in the Department of Virology in the group of prof J.L. Heeney, where she worked on the SIV infection model to study the efficacy of vaccine candidates. Later that same year she started working in the group of dr. T. Kos, dr. E.J. Verschoor and later dr C.S. Rollier, on the hepatitis C virus infection model in chimpanzees. In 2008, Babs started this PhD project at the department of Virology under the supervision of dr G. Koopman, dr.A. Boonstra and prof dr. H.L.A Janssen.

Babs will continue working at the BPRC on the development of non-human primate models for human diseases.

PhD PORTFOLIO

General courses

Course on Laboratory Animal Science (FELASA requirements, level C)	2011
English writing for PhD students	2011
Writing successful grant proposals	2011

Specific courses

NVVI Lunteren Courses-	
Crossing borders	2014
Intracellular logistics and inflammation	2013
The function of APC in health end disease	2012
Features and functions of T-cells in health and disease	2011
Interactions between imm. system and non-imm tissues	2010
Sensing and signalling by the immune system	2009

Seminars and workshops

Euprim Workshop Alternative methods for the use of non-human primates in biomedical research	2014
Research Integrity; Erasmus University	2015

Presentations

Poster: Biomarkers of microbial translocation/immune activation are increased in HCV infected patients with liver fibrosis, but not in HCV infected chimpanzee**, NVVI, Noordwijkerhout.**	2014
Poster: Experimental infection of rhesus macaques and common marmosets with a European strain of West Nile virus**, NVVI, Noordwijkerhout.**	2013
Poster: Serum IP-10 levels correlate with virus load in hepatitis C infected chimpanzees and evidence for novel IL-28B polymorphism. **NVVI, Noordwijkerhout.**	2012
poster: Serum IP-10 levels correlate with virus load in hepatitis C infected chimpanzees and evidence for novel IL-28B polymorphism**., 19th Int HCV meeting, Venice, Italy**	2012
Poster: Hepatitis C Virus envelope protein 1 elicits neutralizing antibodies and protects chimpanzees from persistent infection. **NVVI, Noordwijkerhout.**	2011

Poster: Vaccine induced protection against chronic HCV infection in chimpanzees is associated with CD8+ mediated killing capacity but not with cytokine production. **KeyStone HIV vaccines and viral immunity, Banff, Canada** 2010

Poster: Vaccine induced protection against chronic HCV infection in chimpanzees is associated with CD8+ mediated killing capacity but not with cytokine production. **NVVI, Noordwijkerhout.** 2010

Poster: Hepatitis C Virus envelope protein 1 elicits neutralizing antibodies and protects chimpanzees from persistent infection. **16th Int HCV meeting, Nice, France** 2009

Poster: Vaccine induced protection against chronic HCV infection in chimpanzees is associated with CD8+ mediated killing capacity but not cytokine production**. NVVI, Noordwijkerhout.** 2009

Oral: Patterns of NS3 epitope specific T-cell responses in vaccinated-resolved, versus immunised but persistently infected animals. **UK meeting HCV, Rydall, UK.** 2008

Voluntary work

Editorial board of digital calendar NVVI 2015
Columniste BredaVandaag 2015

(Inter)national conferences

NVVI annual meeting, Kaatsheuvel, The Netherlands 2014
EU WINGS Meeting –Madrid, Spain 2014
Symposium HCV animal models and vaccine development
-Tallinn, Estonia 2013
NVVI annual meeting, Noordwijkerhout, The Netherlands 2013
NVVI annual meeting, Noordwijkerhout, The Netherlands 2012
19th Int. Symposium on HCV and related viruses- Venice, Italy 2012
NVVI annual meeting, Noordwijkerhout, The Netherlands 2011
18th Int. Symposium on HCV and related viruses- Seattle, USA 2011
NVVI annual meeting, Noordwijkerhout, The Netherlands 2009
KeyStone HIV vaccines and viral immunity, Banff, Canada 2010
NVVI annual meeting, Noordwijkerhout, The Netherlands 2010
16th Int. Symposium on HCV and related viruses- Nice, france 2009

LIST OF PUBLICATIONS

Verstrepen BE, Nieuwenhuis IG, Mooij P, Bogers WM, Boonstra A, Koopman, G. Spontaneous and NCR mediated cytotoxicity are effector functions of distinct NK subsets in HCV infected chimpanzees. *Submitted for publication*

Verstrepen BE, Nieuwenhuis IG, Mooij P, Verschoor EJ, Fagrouch Z, Kondova I, Boonstra A, Koopman G. Increased soluble CD14 levels in the absence of liver fibrosis and microbial translocation in hepatitis C virus infected chimpanzees. *Submitted for publication*

Verstrepen BE, Boonstra A, Koopman G. Immune mechanisms of vaccine induced protection against chronic hepatitis C virus infection in chimpanzees. *World J Hepatol 2015 January 27; 7(1): 53-69*

Verstrepen BE, Oostermeijer H, Fagrouch Z, van Heteren M, Niphuis H, Haaksma T, Kondova I, Bogers WM, de Filette M, Sanders N, Stertman L, Magnusson S, Lőrincz O, Lisziewicz J, Barzon L, Palù G, Diamond MS, Chabierski S, Ulbert S, Verschoor EJ. Vaccine-induced protection of rhesus macaques against plasma viremia after intradermal infection with a European lineage 1 strain of West Nile virus. *PLoS One. 2014 Nov 13;9(11):e112568.*

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