

**Basic research for the development
of
hepatitis C vaccine**
C 型肝炎ワクチン開発に向けた基盤研究

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General Abstract

Hepatitis C is a liver disease caused by hepatitis C virus (HCV). HCV can cause chronic hepatitis infection with 80% rate of HCV infected individuals. Chronic hepatitis can lead to cirrhosis and hepatocellular carcinoma. HCV carriers are approximately 185 million people worldwide. The number is still increasing especially in developing countries. Currently HCV treatment can cure approximately 90% of HCV patients but prophylactic and therapeutic vaccines have not yet been available. The development of hepatitis C vaccines is necessary because HCV drugs are extremely expensive, not accessible to all HCV patients. In this point of view, I evaluated safety and efficacy of a novel hepatitis C vaccine candidate and established a novel animal model for evaluating prophylactic and therapeutic effects of vaccine candidates, aiming at developing feasible hepatitis C vaccines as a final goal.

First of all, I injected a novel hepatitis C vaccine candidate into common marmosets (*Callithrix jacchus*) and checked safety of the vaccine candidate, supported by my colleague; Dr. Higashino. The vaccine candidate was composed of inactivated whole-virion prepared from cell culture-generated HCV (HCVcc) and established by my collaborators; Dr. Kato and his colleagues in Department of Virology II, National Institute of Infectious Disease. After vaccination, I evaluated *IFN* γ mRNA expression in peripheral blood mononuclear cells (PBMC) and splenocytes. By immunization with HCVcc with adjuvant (K3-SPG), the expressions of *IFN* γ mRNA in splenocytes were increased after cultivation with HCV core peptides. Vaccination-related abnormality was not detected. Together with the data in terms of antibody responses provided by the collaborators, I concluded that the inactivated HCVcc and K3-SPG may be promising as

a novel hepatitis C vaccine candidate.

The animal model is essential to evaluate the prophylactic and therapeutic effects of hepatitis C vaccine candidates. However the host tropism of HCV is so strict and humans and chimpanzees are only permissive. In order to establish an animal model for evaluating prophylactic and therapeutic effects of hepatitis C vaccine candidates, I hypothesized that chimeric virus constructed by HCV and GB virus B (GBV-B) and New World monkeys (NWMs) can be an appropriate animal model. GBV-B is closely related to HCV and can cause chronic hepatitis in NWMs. I generated a HCV/GBV-B chimera and observed that the chimera persistently replicated in a red-handed tamarin (*Saguinus midas*) for more than two years after the intrahepatic inoculation, although the viral load was relatively low. This result will help establish the novel animal model for developing hepatitis C vaccines on the basis of the HCV/GBV-B chimera in the major framework of HCV genome. This study was published in *Microbiology and Immunology* 60, 26-34 in 2016.

Although this model can be useful for evaluating hepatitis C vaccine candidates, it is still need to improve replication efficiency and virulence. In order to seek factors for higher replication efficiency and virulence, I analyzed that the differences of humoral and cellular immune responses in acute and chronic phases of GBV-B infection. The induction of anti-E2, core and NS3 antibodies in chronically GBV-B-infected monkeys was much delayed to reach plateau irrespective of the levels of plasma viral RNA which were theoretically sufficient to induce efficient antibody responses. Furthermore, NK cells expressing cytotoxic markers (CD3⁺ CD16⁺ CD56^{dim} CD335⁺ CD159a⁺) increased in the cell number in course of the acute clearance. On the other hand, the rate of the cytotoxic NK cells in the chronically infected monkey remained low as much as that in

naïve and the GBV-B-cured monkeys. These results suggest that the host factors which associated with outcome of GBV-B infection may be the induction of antibodies and cytotoxic NK cells.

To apply these findings to an animal model, I sought to decline the cellular immunity by depleting cytotoxic T lymphocytes (CD8⁺ T cells) in marmosets. Here I injected a novel marmoset CD8-specific monoclonal antibody (mAb) into common marmosets and observed that drastic and specific depletion of CD8⁺ T cell subset. It is hoped that this method will contribute to establish an animal model which shows higher replication efficiency and virulence of the HCV/GBV-B chimera. This study was published in Immunology Letters 154, 12-17, 2013 in collaboration with Dr. Yoshida. We are contributed equally.

In conclusion, to establish an animal model for evaluating prophylactic and therapeutic effects of hepatitis C vaccine candidates, I generated the HCV/GBV-B chimera and inoculated it into a tamarin. The chimera showed persistent replication *in vivo*, which implies to be an appropriate animal model. Furthermore, I clarified that the induction of antibodies and cytotoxicity of NK cells are one of the factors to persist GBV-B infection. Applying these findings, I established the method to decline cellular immunity in NWMs by administrating novel marmoset CD8-specific mAb for improving the animal model. I believe that these knowledges found in my study are newly and critical for hepatitis C vaccine study and will contribute to effectuate development of hepatitis C vaccines in the future and lead to a breakthrough in hepatitis C study.

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Abbreviations

AIDS	acquired immune deficiency syndrome
ALB	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
Alum	Aluminium hydroxide
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
AST	aspartate aminotransferase
BUN	blood urea nitrogen
CHV	canine hepacivirus
CLDN1	claudin 1
ConA	concanavalin A
CpG ODNs	CpG oligodeoxynucleotides
CRP	C-reactive protein
CTL	cytotoxic T lymphocytes
DAA	direct-acting antivirals
Dnase	deoxyribonuclease
EIA	enzyme immunoassay
FBS	fetal bovine serum
FFU	focus forming units
FPV	fowlpoxvirus
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBV-B	GB virus B
GBV-Bpp	GBV-B pseudo particles
γ GTP	γ -glutamyl transpeptidase
HCC	Hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	cell culture-generated HCV
HCV-LPs	HCV-like particles
HCVpp	HCV pseudo particles
HDL	high-density-lipoprotein
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IFN α	interferon α
IFN γ	interferon γ
IgG	immunoglobulin G
IRES	internal ribosome entry site

K3	K type of CpG
LDH	lactate dehydrogenase
LDL	low-density-lipoprotein
LDLR	low-density-lipoprotein receptor
M.O.I.	multiplicity of infection
mAb	monoclonal antibody
MHC	major histocompatibility complex
miR-122	micro RNA-122
MS	multiple sclerosis
MVA	modified vaccinia virus Ankara strain
NAb	neutralizing antibodies
NK	Natural Killer
non-A	non-hepatitis A
non-B	non-hepatitis B
NPHVs	non-primate hepaciviruses
NWMs	New World monkeys
OCLN	occluding
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pegIFN	pegylated IFN
PLT	platelet count
RA	rheumatoid arthritis
RBC	red blood cells
RdRp	RNA-dependent RNA polymerase
SCID	Severe Combined Immunodeficiency
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
SNP	single nucleotide polymorphism
SPG	schizophyllan
SR-B1	scavenger receptor B1
SVR	sustained virological response
TBS-T	Tris-buffered saline containing Tween-20
TP	total protein
UTR	untranslated regions
VLDL	very-low-density-lipoprotein
WBC	white blood cells

Notes

Contents of the present thesis were published in the following articles.

1. **Suzuki S, Mori K, Higashino A, Iwasaki Y, Yasutomi Y, Maki N, Akari H.** 2016.
Persistent replication of a hepatitis C virus genotype 1b-based chimeric clone
carrying E1, E2 and p6 regions from GB virus B in a New World monkey.
Microbiol Immunol **60**:26-34
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2. **Yoshida T, Suzuki S, Iwasaki Y, Kaneko A, Saito A, Enomoto Y, Higashino A,
Watanabe A, Suzuki J, Inoue K, Kuroda T, Takada M, Ito R, Ito M, Akari H.**
2013. Efficient *in vivo* depletion of CD8⁺ T lymphocytes in common marmosets by
novel CD8 monoclonal antibody administration. Immunol Lett **154**:12-17.
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General Introduction

Human being has struggle with infectious diseases caused by numerous pathogens which try to invade our bodies for long time period. Nowadays, many kinds of treatments against infectious diseases have developed and saved us from pain and death of diseases, thanks to past scientist's efforts and animal's sacrifice. However still many refractory infections threat our health.

Acute hepatitis C virus infection

Hepatitis C has been and still now is one of the refractory infections although many effective drugs have been developed. Hepatitis C mostly affects human liver, caused by hepatitis C virus (HCV) infection. HCV causes acute hepatitis and approximately 75-85% of HCV infected individuals progress to chronic hepatitis (1). Limited proportion of individuals appears to be resistant to HCV infection and remain seronegative and uninfected despite high-risk of exposure, such as injection drug users who share drug injection equipment (2-4). Acute HCV infection is scarcely diagnosed, because the majority of acutely infected patients are asymptomatic. Only 20-30% of adults with acute HCV infection may develop clinical symptoms (1). The onset of symptoms may occur from 3 to 12 weeks after HCV exposure. Symptoms are non-specific, including malaise, weakness, anorexia and jaundice. One of hepatitis markers, serum alanine aminotransferase (ALT) level increases 2-8 weeks after exposure and often reaches to the levels ten-fold or more than the one before infection. The level of HCV RNA rises rapidly a few weeks after exposure, followed by reaching peaks levels at around 10^{5-7} IU/ml, shortly before the peak of serum ALT and onset of

symptoms (1, 5). The antibody against HCV becomes positive 1-3 month after infection (1). Acute HCV infection can be severe but fulminant liver failure is rare (6, 7).

Chronic hepatitis C virus infection

Chronic hepatitis C is marked by the persistence of HCV RNA in the blood for at least six months after onset of acute infection (1, 8). The causes for chronic hepatitis C include many factors such as age, gender, ethnicity and genetic background. Generally, individuals who are old and male develop chronic hepatitis C with high rate. And African-Americans appear to have a higher rate of chronic HCV infection than Caucasians and Hispanic whites (1). Majority of onset to chronic hepatitis C occurs in the absence of symptoms (8). Fibrosis is the formation of excess fibrous connective tissue in a reactive process to inflammation in liver. Sixty-nine percent among the chronically infected group had increased ALT values (8). Although the precise percentage is controversial, it is reported that 10-20% (8) or 16% (9) of chronic hepatitis C individuals develop cirrhosis within 20 years after infection. Cirrhosis is severe scarring of the liver, although generally occurs without symptoms and the presence of cirrhosis may remain unrecognized until the liver becomes sufficiently compromised to advance to overt hepatic decompensation (8). Most of HCV-infected individuals with cirrhosis and without any treatments may develop hepatocellular carcinoma (HCC) at an annual rate of 1-4%. Higher estimates in the range of 5-7% have been reported from Japan. This may be because higher proportion of elderly patients infected with HCV in Japan, since older age accelerates the development of HCC (1). Finally more than 350,000 people deacease each year in the world dues to HCV-related diseases including cirrhosis and HCC according to 2002 worldwide mortality estimates (10, 11).

Prevalence of HCV in the world and Japan

Globally, an estimated 130-170 million person (2-3% of world's population) are infected with HCV (10, 12). The prevalence of HCV infection varied considerably within and between regions. HCV infection rates are higher ($\geq 3\%$) in many countries in eastern Europe and Latin America, countries of the former Soviet Union and certain countries in Africa, Middle East, and South Asia (10, 12-17). Egypt is reported to have the highest rate of hepatitis C in the world (estimated at $>10\%$) (13, 18).

In Japan, two million people are HCV carriers (19). The highest cause of deaths is malignant neoplasm, and HCC is the 4th or 5th highest cause of cancer-related deaths in male or female, respectively, according to the report of the Ministry of Health, Labour and Welfare in 2014. It is reported that 70-80% of HCC are associated with HCV, approximately 30,000 patients died of HCV in 2002 (20).

The route of transmission

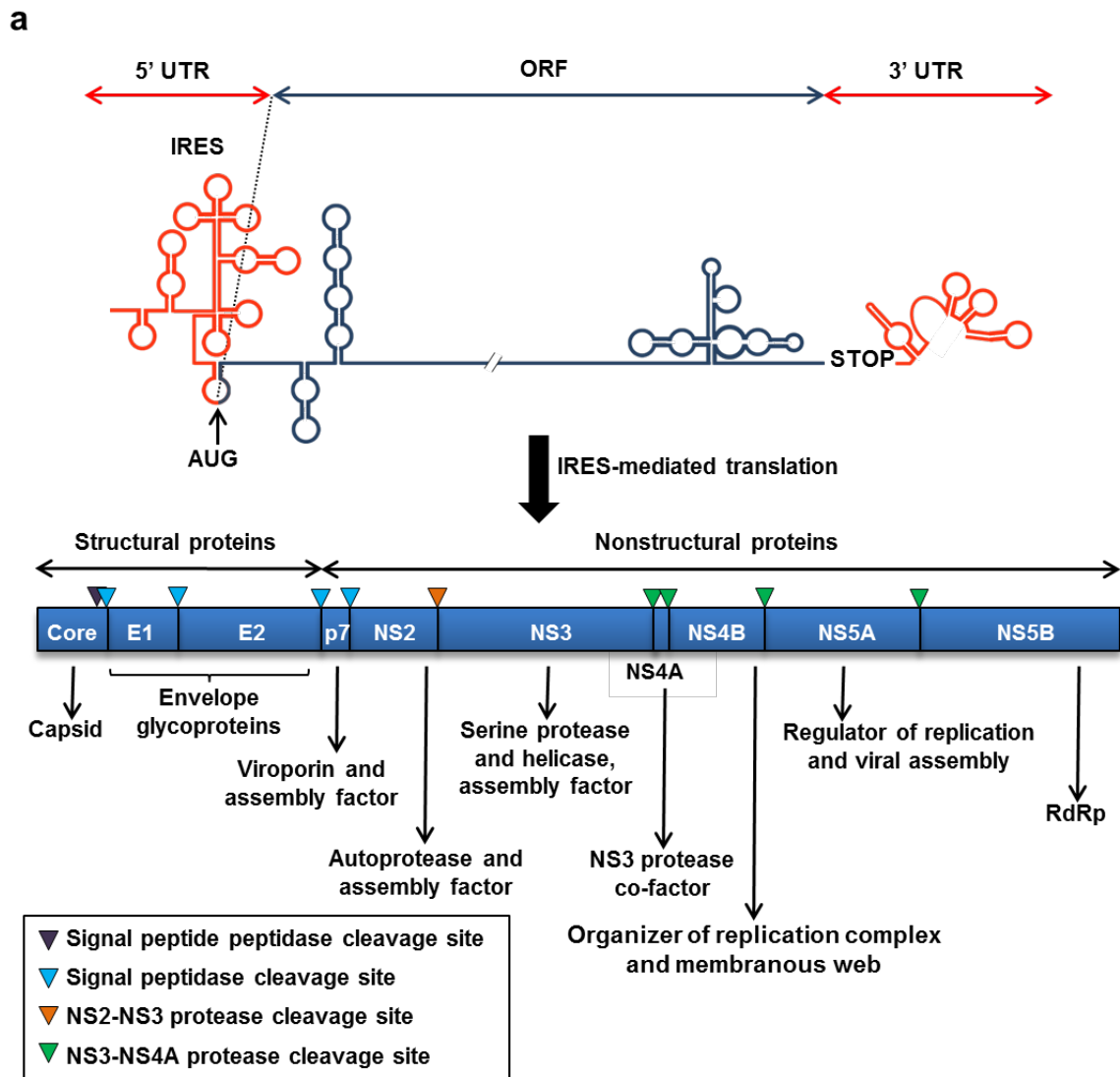
HCV is blood-borne virus and can be transmitted by contaminated blood transfusion or re-use of infected medical devices like injection equipment when mass vaccination or injecting drugs (12, 13). Transmission of HCV infection through perinatal and sexual exposures occurs with much less efficiency than other routes of transmission (14). In developed countries, donated blood is routinely screened for HCV and the risk of transmission by blood transfusion is rare. Now HCV is predominantly transmitted through injection drug use (10, 18, 21). On the other hand, in developing countries, injection drug use also can be an important risk factor for HCV transmission (22, 23), but the most common modes of transmission are associated with healthcare (18, 24, 25).

Unsafe injections in healthcare settings are a leading cause of HCV transmission worldwide, resulting in two million new HCV infections per year (24). Recipients of blood and blood products also are at risk of HCV infection. In Japan, contaminated blood transfusion and re-use of injection equipment in the course of mass vaccination mainly caused spread of HCV infection. As well as mass vaccination, re-use of injection equipment for parenteral antischistosomal therapy with antimony was also responsible for the spread of HCV infection (25). This is also the reason why Egypt has the highest HCV prevalence in the world (25-27). Furthermore, fibrinogen concentrate, treatment for obstetric bleeding, was also contaminated with HCV, which raised the number of HCV-infected patients until 1988. As a result, an estimated 10,000 cases of hepatitis C infection were attributed to the use of fibrinogen (28). Nowadays, the risk of hepatitis C by iatrogenic reason is quite low in Japan.

Virology of HCV: life cycle, genotypes and experimental systems

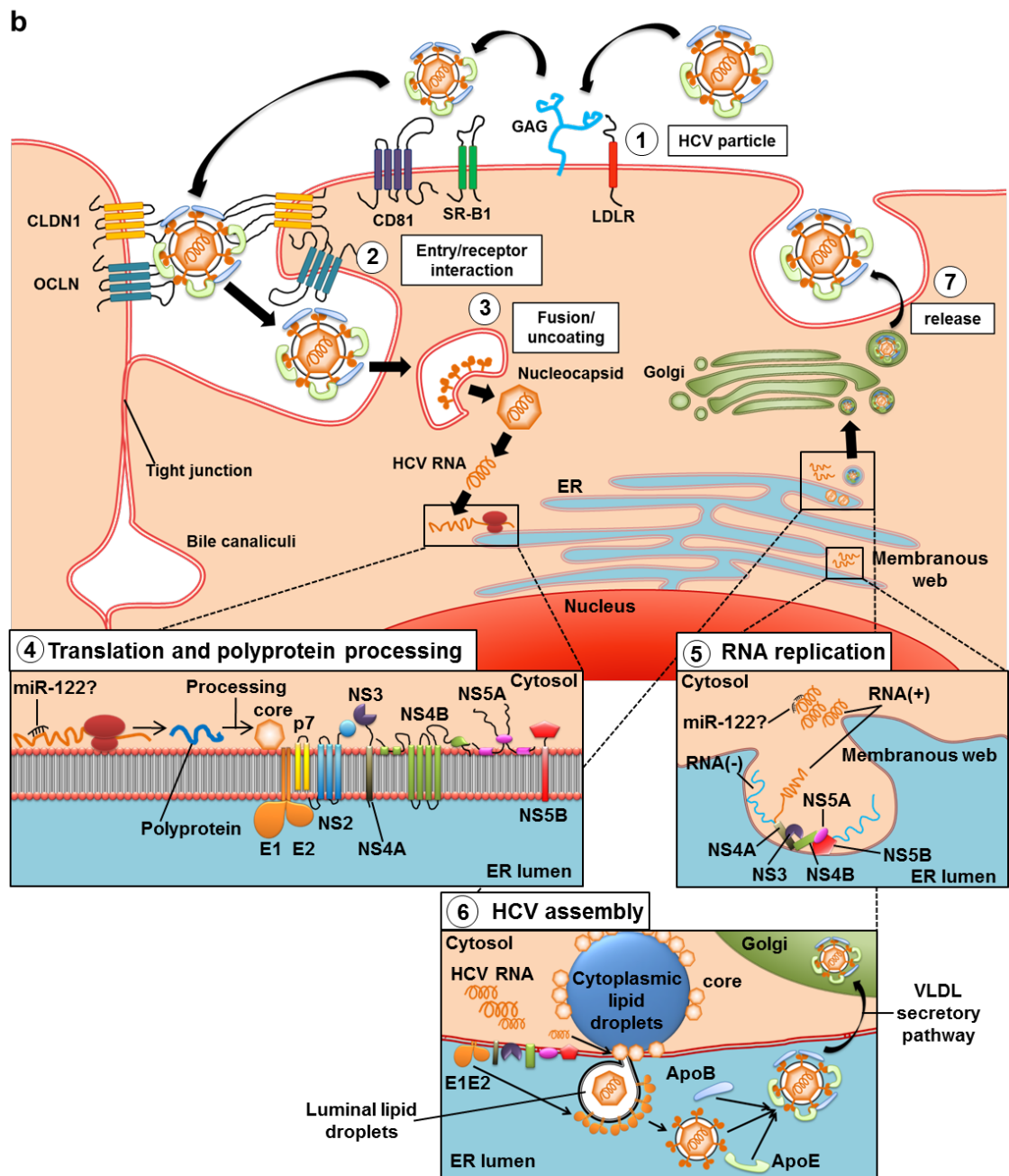
HCV is an enveloped, positive-sense single-stranded RNA virus of the family *Flaviviridae*, Genus *Hepacivirus*. HCV was identified as a major causative agent of non-hepatitis A (non-A), non-hepatitis B (non-B) hepatitis in 1989 (29). The genome of HCV is approximately 9.6 kilobases in length, constructed by open reading frame (ORF) and untranslated regions (UTR); 5'UTR and 3'UTR. After HCV invaded into a body, HCV is physically associated with very-low-density-lipoprotein (VLDL), low-density-lipoprotein (LDL) and high-density-lipoprotein (HDL) (30). HCV enters into hepatocytes by binding to tetraspanin CD81, scavenger receptor class B type I (SR-B1) and tight junction proteins, such as claudin 1 (CLDN1) and occluding (OCLN) (30-32). Once HCV is internalized into endosomal vesicles, the acidic pH environment

results in fusion of viral and endosomal membranes. The viral RNA is then released into the cytoplasm, whereupon it undergoes translation, resulting in a single viral polyprotein. This polyprotein is processed into ten individual proteins by viral enzymes (NS2/3 and NS3/4A) and cellular enzymes (signal peptidase and signal peptide peptidase). Ten proteins are constructed by structural protein: core, E1, E2, and nonstructural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B (32, 33) (Supplementary Fig. 1a). Replication occurs at an endoplasmic reticulum membrane-derived replication complex (membranous web), which includes the lipid droplet and NS4A-NS5B. At membranous web, a positive-strand RNA is copied by NS5B RNA-dependent RNA polymerase (RdRp) into a negative-strand RNA intermediate, which in turn serves as a template for the new viral genomic RNA (Supplementary Fig. 1b). This replication also required host factors such as protein involved in lipid metabolism, as well as micro RNA-122 (miR-122) although this is not yet fully understood. The newly synthesized viral RNA is assembled into new lipidated lipoviral particle by Golgi apparatus and subsequently released by the cell (32, 34, 35).



Supplementary Fig. 1 Schematic diagram and life cycle of HCV

(a) The HCV genome and polyprotein processing. The HCV RNA genome (top) contains one long ORF (blue) flanked by 5' and 3' UTRs (red). Internal ribosome entry site (IRES)-mediated translation of the ORF leads to a polyprotein (bottom) that is co- and post-translationally processed into ten viral proteins. The maturation process of the core protein involves a cellular signal peptide peptidase cleavage of a C-terminal signal peptide (purple triangle) and cleavage from E1 by the cellular signal peptidase, which also cleaves E1, E2 and p7 from the polyprotein (blue triangle). In an autocleavage mechanism requiring two identical molecules to make up the composite active site, the NS2-NS3 protease cleaves itself (orange triangle). The NS3 protease located in the first one-third of NS3, assisted by its membrane-bound co-factor, NS4A, cleaves the remaining proteins NS3, NS4A, NS4B, NS5A and NS5B (green triangle). The functions of the individual HCV proteins are indicated. (35)



Supplementary Fig. 1 (continue)

(b) Life cycle of HCV. Interaction of extracellular HCV particle (1) with cellular surface receptors initiates the entry process (2). After pH-dependent fusion and uncoating (3), the incoming HCV genome is translated and the resulting polyprotein processed (4). Replication takes place in membranous web (5). The contribution of miR-122 binding to the HCV genome is not yet fully understood. In the assembly and release process (6), core protein is transferred from cytoplasmic lipid droplets. HCV virion morphogenesis is coupled to the VLDL pathway, and the particles are produced as lipoviroparticles (7). (35)

HCV strains are classified into seven recognized genotypes 1-7 on the basis of phylogenetic and sequence analyses of whole viral genomes (36, 37). HCV strains belonging to different genotypes differ at 30-35% based on nucleotide sequences. Within each genotype, HCV is further classified into 67 confirmed and 20 provisional subtypes. Strains that belong to the same subtype differ at <15% of nucleotide sequences (36, 38). In Japan, 70% of HCV carriers are infected with HCV genotype 1b, 20% with genotype 2a, and the remainder with genotype 2b or other genotypes (39). Globally, genotype 1 is the most prevalent (46.2% of all HCV cases) and genotype 3 is the next most prevalent (30.1%) (36).

Since discovery of HCV in 1989, the lack of a cell culture system for the production of infectious HCV particles represented a major obstacle for studying the viral life cycle and for developing therapies. Historically, patient-derived HCV isolates failed to initiate productive infection in cell culture. Until 2005 when the cell culture system was developed, surrogate systems were developed to dissect individual steps of HCV life cycle (31). Soluble E2 ectodomain is a soluble form of the HCV glycoprotein E2, the first tool which is used to search for HCV receptors in 1997 (40). HCV subgenomic replicon system was established in 1999, which was the minimal set of HCV proteins required to initiate and maintain HCV replication in human cells (41). Production of HCV pseudo particles (HCVpp) is a major breakthrough for investigating the process of HCV entry in 2003, which consists of defective retroviral particles expressing HCV envelope glycoproteins on their surface (42, 43). After that, in 2005, an only genotype 2a HCV isolate (JFH-1) from a Japanese patient with fulminant HCV-associated hepatitis, which is able to initiate low-level infection in Huh-7, a human hepatoma cell line, paved the way to studying the complete HCV life cycle in cell culture (44). This

model system enabled for the first time studies of HCV assembly, egress, the characterization of biophysical and ultrastructural properties of the HCV particles. The cell culture-generated HCV (HCVcc) is infectious in chimpanzees (*Pan troglodytes*) and human liver-chimeric mice (45), facilitating the evaluation of vaccine candidates *in vivo*. However, *in vivo* systems of HCV are just beginning of study compared to other virus, further analyses are necessary, such as cell culture systems of other genotypes.

Treatments of hepatitis C

Treatments of hepatitis are divided into symptomatic therapy and causal therapy. The symptomatic therapy includes taking ursodeoxycholic acid and phlebotomy to inhibit ALT elevation and iron overload which are believed to enhance the progression of HCC (46, 47). I detailed causal therapy in 1.2. Introduction of Chapter 1. Briefly, in 1992, interferon (IFN) treatment was approved first, and then the treatment using pegylated IFN (pegIFN) which was IFN modified by the attachment of a 40 kilo Dalton branched-chain polyethylene glycol moiety showed higher SVR than IFN treatment (48). The treatment of pegIFN and ribavirin, a guanosine analog used as one of anti-viral drugs, has been the mainstay of therapy for more than 25 years and still one of the standards of therapy. And now IFN-free, all-oral regimen of direct-acting antivirals (DAAs) such as Sofosbuvir (SOF) with high sustained viral response (SVR) have been approved. SVR is an index of recovery from HCV infection, defined as undetectable HCV RNA 12 or 24 weeks after completion of therapy. However, DAAs are inaccessible so far to most HCV carriers around the world especially in developing countries where the number of HCV carrier is still increasing since the cost of DAAs is extremely expensive. The treatment of all HCV carriers will cost on the region of

US\$ ten trillion (49). Also in Japan, these DAAs are so expensive, but burden of the cost is not so heavy to patients because it is borne by health cost insurance and medical expenses subsidy. On the other hand, in developing countries which have poor bailout, burden of the cost is so heavy to patients that effective DAAs are not accessible. Therefore, prophylactic and therapeutic vaccines are anticipated to be developed but are still unavailable. This is because there are no animal models to evaluate prophylactic and therapeutic effects of vaccine candidates as well as the delay of basic study of HCV.

Animal model for HCV study

The critical obstacles are the delay of basic HCV study and strict host specificity of HCV. HCV can infect only humans and chimpanzees. Chimpanzees are naturally susceptible to HCV infection and their use in HCV research has greatly advanced our understanding of HCV study. In 1997, the development of HCV molecular clones that were infectious in chimpanzees was an important initial breakthrough although these viral genomes did not produce viral particles in cell culture (50, 51). Since 1997 until 2013 when chimpanzee research was banned in most countries due to ethical concerns, chimpanzees were the most important *in vivo* model for HCV study (31). Prophylactic and therapeutic effects in vaccine candidates cannot be evaluated without animal model which are susceptible to HCV infection. Instead of chimpanzees, surrogate animal models which can evaluate vaccine candidates are indispensable.

Treeshrews (*Tupaia belangeri*) were shown to be susceptible to HCV infection (52). HCV caused mild hepatitis during acute infection but didn't cause chronic infection in the animals (53). In addition, it is difficult to study host responses and to breed them for widespread use in HCV research (31).

Although mice are naturally resistant to HCV entry and replication, genetically humanized mouse model expressing human CD81 and OCLN via adenoviral gene delivery or by transgenic expression, were shown to support HCV entry (54). This model can be suitable to evaluate vaccine candidates, but no intracellular accumulation of viral RNA, indicating that viral RNA replication is insufficiently recapitulated in murine cells (31, 54, 55). Human liver chimeric mouse models are obtained by transplanting normal human hepatocytes into Severe Combined Immunodeficiency (SCID) mice carrying a plasminogen activator transgene (Alb-uPA mice), developed prolonged HCV infection with high viral titers after inoculation with human serum containing infectious HCV (56). This model is suitable for studying whole HCV lifecycle. However the recipient mice were immunodeficient mice which lack B cell, T cell as well as NK cell responses to facilitate human graft maintenance (31), and therefore this model is not suitable for evaluating hepatitis C vaccine candidates.

Virology of GB virus B (GBV-B)

GBV-B is an enveloped, positive-sense single-stranded RNA virus of the family *Flaviviridae*, Genus *Hepacivirus*, closely related to HCV. Historically, in attempt to isolate and identify the etiologic agents responsible for non-A, non-B hepatitis, Deinhardt et al. inoculated white lipped tamarin (*Saguinus fuscicollis*), hairy faced tamarin (*Saguinus nigricollis*), crested, barefaced and cotton top tamarins (*Saguinus* (*Oedipus*) *oedipomidas*) with sera from patients with severe hepatitis (57, 58). The serum from a surgeon whose initials were GB, was found to cause hepatitis in four challenged tamarins. Serial passages of the infected tamarin sera also caused hepatitis. The 11th passage of this serum was demonstrated to be infectious in most tamarins. Two

viruses were identified from the serum of tamarin infected with the 11th passage serum and were termed GBV-A and GBV-B (59). Subsequent study showed that GBV-A did not cause hepatitis but could persistently infects New World Monkeys (NWMs) without obvious disease symptom (60). This 11th passage serum was found to be non-infectious in chimpanzees (61). Tamarins and marmosets were not infected by HCV (62, 63). Even the differential infection profiles between HCV and GBV-B could prove helpful in grasping the immune evasion strategies of HCV. Both similarities and the contrasts of the GBV-B model with the HCV infection can serve to broaden the knowledge of HCV pathogenesis (58).

Although natural host of GBV-B has not been unveiled, experimental infection induces acute and chronic hepatitis in small NWMs such as red-handed tamarins (*Saguinus midas*), common marmosets (*Callithrix jacchus*) and owl monkeys (*Aotus trivirgatus*) (58, 64-69). GBV-B has been used as a surrogate model for HCV infection in various studies to understand pathogenesis and to demonstrate candidate antiviral activity. GBV-B causes acute infection in tamarins as well as HCV in humans (70). Experimental GBV-B infection induces viremia and mild acute hepatitis approximately for ten weeks. Some groups reported that GBV-B infected-tamarin caused persistent viremia (68, 69, 71). And one of that groups reported that tamarins inoculated synthetic GBV-B RNA developed high-titer viremia and one of them remained viremia for over two years (68). In previous studies of my laboratory, it is demonstrated that marmosets experimentally challenged with GBV-B developed long-term chronic infection with fluctuated viremia, recurrent increase of ALT, plateaued titers of the antiviral antibodies (66). Histopathological analysis of liver necropsy samples demonstrated that diffuse and abundant fibrosis. These features were comparable to chronic hepatitis C in humans.

These results demonstrated that GBV-B could induce chronic hepatitis C-like disease in marmosets and tamarins.

Recently, Canine hepatitis virus (CHV), Equine hepatitis virus, termed as non-primate hepatitis viruses (NPHVs), have been identified as new members of hepatitis viruses from dogs, horses and African cattle sera (58, 72-77). Novel NPHVs are also found from European bank voles and South African four-striped grass mice (78). CHV is the closest to HCV phylogenetically, can be animal models for HCV study but NPHVs are the closest to humans genetically and immunologically, GBV-B-infected NPHVs is the most appropriate for hepatitis C vaccine study. The use of non-human primates as experimental animal models is highly effective for research on human diseases. Non-human primates and humans share comparable immune systems as compared with mice and are suitable for the evaluation of innate and adaptive immune responses against pathogenic viruses (79, 80). On the other hand, there are also several issues with chimpanzees and macaques. The use of the chimpanzees is limited by ethical and financial restrictions as described above (81-84). Tamarins and marmosets, small NPHVs, have several advantages as an experimental animal model. The small-sized NPHVs make it easier to handle and reduces maintenance costs (85). Recently, it has been reported that the marmoset model is a very useful tool in investigating multiple sclerosis (MS), rheumatoid arthritis (RA) and Parkinson's disease (86-88). Moreover, the marmosets have an immune system similar to that of humans and they are suitable for the evaluation of innate and adaptive immune responses against viruses which efficiently replicate in the marmosets (89-92).

Purpose of this study

Taking these backgrounds into consideration, I hypothesized that chimeric virus of GBV-B and HCV can infect NWMs and cause hepatitis C-like hepatitis. Furthermore, HCV/GBV-B chimera-infected NWMs can be an appropriate animal for evaluating prophylactic and therapeutic effect of hepatitis C vaccine candidates. This animal model will be able to give us important knowledges for HCV study such as mechanisms of chronic infection, and it can evaluate vaccine candidates as a preclinical study and select useful one to proceed clinical trials. The final goal of my study is to develop hepatitis C vaccines by using HCV/GBV-B chimera-infected NWMs and to prevent prevalence of HCV infection by the broadly available hepatitis C vaccines. I believe that this study will contribute the breakthrough in hepatitis C study. In my thesis study, I performed four themes with a goal of development of hepatitis C vaccines.

In 1st chapter, I aimed to evaluate a novel hepatitis C vaccine candidate in collaboration with Dr. Kato and his colleagues in Department of Virology II, National institute of Infectious Diseases. The hepatitis C vaccine candidate was composed of inactivated whole-virion prepared from HCVcc together with adjuvant (Alum or K3-SPG). After I injected them into ten common marmosets and checked safety of the candidates, supported by my colleague; Dr. Higashino, I evaluated cellular immune response by measuring *IFN γ* mRNA expression in peripheral blood mononuclear cells (PBMC) and splenocytes, while the collaborators evaluated antibody response. As a result, I found that the combination of inactivated HCVcc and K3-SPG induced effective cellular immune responses against HCV antigen without any obvious side effects. Together with the data in terms of antibody responses provided by the collaborators, I concluded that the inactivated HCVcc and K3-SPG may be promising as a novel hepatitis C vaccine candidate. This work is in preparation of the manuscript for

submission.

In 2nd chapter, I established a novel HCV/GBV-B chimera and evaluated the replication ability *in vitro* and *in vivo* since an animal model on the basis of new concept is essential to evaluate the prophylactic and therapeutic effects of hepatitis C vaccine candidates considering the present situation that no feasible animal model is available to evaluate hepatitis C vaccine candidates. I observed that the chimera persistently replicated in a red-handed tamarin for more than two years after the intrahepatic inoculation, although the viral load was relatively low. This is the first finding showing that the HCV/GBV-B chimera in the major framework of HCV genome is replication-competent *in vivo*. This work is published in Microbiology and Immunology 60, 26-34, 2016.

In 3rd chapter, I aimed to improve replication efficiency and virulence of this chimera for the purpose of evaluating prophylactic and therapeutic effects of the vaccine candidate. I hypothesized that some host factors restrict replication efficiency and the same factors also prevent viruses from causing chronic infection. However the use of periodical samples from HCV-exposed individuals without receiving anti-viral treatments is virtually unavailable. In this point of view, I analyzed that the differences of humoral and cellular immune responses in acute and chronic phases of GBV-B infection and sought host factors regulating chronic infection. The induction of anti-E2, core and NS3 antibodies in chronically GBV-B-infected monkeys was much delayed to reach plateau irrespective of the levels of plasma viral RNA which were theoretically sufficient to induce efficient antibody responses. Furthermore, NK cells expressing cytotoxic markers (CD3⁻ CD16⁺ CD56^{dim} CD335⁺ CD159a⁺) increased in the cell number in course of the acute clearance. On the other hand, the rate of the cytotoxic NK cells in

the chronically infected monkey remained low as much as that in naïve and the GBV-B-cured monkeys. These results suggest that the host factors which associated with outcome of GBV-B infection may be timing of the induction of antibodies and cytotoxic NK cells. This work is in preparation of the manuscript for submission.

In 4th chapter, I sought to decline the cellular immunity by depleting CD8-positive cytotoxic T lymphocytes (CD8⁺ T cells) in common marmosets to apply these findings to an animal model in which chimera replicates efficiently. Of note, the previous reports support my hypothesis because CD8⁺ T cells-depleted chimpanzees showed prolonged HCV infection (93). CD4⁺ or CD8⁺ T cells-depletion prevented HCV clearance and clinical recovery (94, 95). CD8⁺ T cells are a vital component of the adaptive immune response and are crucial to the control and clearance of intracellular pathogens. These cells play critical roles in purging acute infection, limiting persistent infection, and conferring life-long protective immunity. In order to directly clarify the pivotal role of CD8⁺ T cells, *in vivo* depletion of CD8⁺ T cells by administration of a CD8-specific monoclonal antibody (mAb) is a straightforward technique. In fact, it has been established in Old world monkeys (93, 95-100). However, the anti-CD8 mAb which is specific for NWMs and is capable of depleting CD8 T cells *in vivo* has not been reported. Recently, Dr. Ryoji Ito and Dr. Mamoru Ito in Central Institute for Experimental Animals established hybridoma anti-marmoset mAb against marmoset CD8⁺ T cells producing and generated a sufficient amount of the mAb to inject several marmosets (85). They kindly gave me the mAb and I injected an anti-marmoset CD8 mAb into marmosets and observed that drastic and specific depletion of CD8⁺ T cell subset for the first time. It is hoped that this method will contribute to establish an animal model which shows higher replication efficiency and virulence of HCV/GBV-B

chimera. The study of depletion of CD8⁺ T cells is collaborated with Dr. Yoshida and published in Immunology letters 154, 12-17, 2013. We are contributed equally.

I believe that these knowledges found in my study are newly and critical for hepatitis C vaccine study and will contribute to effectuate development of hepatitis C vaccines in the future and lead to a breakthrough of hepatitis C study.

Chapter 1

**Evaluation of safety and efficacy of a novel hepatitis C vaccine
candidate composed of inactivated hepatitis C virus particle**

1.1. Abstract

Although novel direct-acting antivirals (DAAs) agents have increased the number of patients who respond to treatment, it is still important to develop the vaccine to prevent the infection of this virus because the cost of the new DAAs is extremely high and the number of HCV-infected individuals is still increasing in the world. However hepatitis C vaccine is not available so far due to lack of appropriate animal model and delay of basic research of HCV. In this study, we aimed to establish the novel vaccine of hepatitis C virus (HCV) by exploiting the cell culture-generated HCV (HCVcc), and validated the efficiency of neutralizing antibodies (NAb) and *IFN* γ mRNA induction in non-human primate model in collaboration with Dr. Kato and Mr. Yokokawa et al. in Department of Virology II, National institute of Infectious Diseases. This is the first trial that inactivated HCVcc generated by transfecting the full-length RNA of J6/JFH-1 chimeric virus (genotype 2a/2a) was inoculated into common marmosets (*Callithrix jacchus*) as a vaccine candidate. The technique to generate HCVcc particle enough to inoculate into marmosets has not been developed so far. Common marmosets were subcutaneously and intramuscularly immunized with purified and inactivated HCVcc in combination with either of two types of adjuvants, i.e., authentic adjuvant, Alum or newly developed potent adjuvant, K3-SPG (schizophyllan conjugated CpG). Vaccination-related abnormality by immunization was not detected in blood tests in all vaccinated animals. Although it has been shown that NAb is associated with the control of the viral persistence, it is also reported that only induction of NAb may not be able to control HCV infection perfectly. Taken together, the induction of cellular immune response as well as humoral immune response should be the key to develop the efficient

hepatitis C vaccines. Hence, I evaluated the expression of *IFN γ* mRNA in splenocytes to investigate activation of cellular immune response. The expression was increased after stimulation with HCV core peptides (genotype 2a) in marmosets which were inoculated HCVcc and K3-SPG. In addition, Mr. Yokokawa investigated that induction of antibodies and NAb. HCV specific antibodies were detected and reacted to E2 and core of J6 (genotype 2a) and TH (genotype 1b) strains. Purified immunoglobulin G (IgG) from these serum samples indicated the neutralizing activity *in vitro* HCV infection system with J6/JFH-1 (genotype 2a/2a) and TH/JFH-1 (genotype 1b/2a) chimeric viruses. The combination of HCVcc and K3-SPG worked as the potent and safe vaccine of HCV in non-human primate model.

1.2. Introduction

Effective drugs against Hepatitis C virus (HCV) have recently been developed. In 1992 interferon α (IFN α) treatment for 24 weeks was approved first, and then the treatment using pegylated IFN (pegIFN) which is modified by the attachment of a 40 kilo Dalton branched-chain polyethylene glycol moiety showed higher SVR than IFN treatment because pegIFN is more stable than unmodified IFN α in the body (48). The treatment of pegIFN and ribavirin which is one of anti-viral drug and inhibits viral RNA synthesis treatment has been the mainstay of therapy for more than 25 years. However the rate of SVR associated with pegIFN/ribavirin combination therapy remained below 50% or less than 30% among patients who have HCV genotype 1 infection and certain baseline characteristics, such as advanced fibrosis, diabetes or coinfection with the human immunodeficiency virus (HIV) (101-106). Since 2011, the combination treatment of NS3/4A protease inhibitor, Telaprevir which is one of direct-acting antivirals (DAAs) and pegIFN/ribavirin was approved and showed high SVR rate in Japan. As the combination treatment with pegIFN/ribavirin, Simeprevir or Vaniprevir were also approved since 2013, 2014, respectively. In addition, IFN-free treatments using all-oral regimen of DAAs with high SVR have been approved. The combination regimen using Daclatasvir/Asunaprevir, NS5A inhibitor and NS3/4A protease inhibitor, has started in September 2014 in Japan. SVR rate of NS5B polymerase inhibitor, Sofosbuvir (SOF) which is one of IFN-free DAAs, is over 90% in the study of genotype 2. Occurrence of resistance mutation against SOF treatment *in vivo* appeared rare (107). However it is not feasible to administrate DAAs to most HCV carriers around the world especially in developing countries where still the number of HCV carrier is increasing since the cost

of DAAs is extremely expensive. The treatment of all of HCV carriers will cost on the region of US\$ ten trillion (49). In Japan, it is planning that the prescription charge of SOF will be set over 60,000 yen per one pill. The period of the treatment is 12 weeks, the total cost will be approximately five million yen per individual. Although there are health cost insurance and medical expenses subsidy in Japan, developing countries have poor bailout. The burden of the cost is too heavy for patients living there to obtain the drug.

Therefore, the development of prophylactic and therapeutic vaccines with lower cost is necessary. Recent hepatitis C vaccine candidates in clinical trials are ongoing but unfortunately no hepatitis C vaccine has been approved yet for practical use. So far all the hepatitis C vaccine candidates in clinical trials employed different delivery approaches such as viral vector using adenovirus, DNA vector coding core-E2 or NS3/4A, recombinant proteins, peptides and HCV-like particles (HCV-LPs) containing core-E2. Immunization by recombinant E1E2 protein in phase I clinical trial, induced envelope-specific CD4⁺ T cells but induced antibodies did not show neutralization in all subject (108). On the other hand, recombinant E1E2 proteins-inoculated chimpanzees induced neutralizing antibodies (NAb). The induced NAb seemed to at least partially protect them from HCV infection (109, 110). Basically, the anti-viral effect induced by recombinant proteins is due to the neutralizing activity by NAb, but the epitopes neutralized by recombinant proteins may mutate and escape from NAb after the viral replication *in vivo*. The adenoviral vector expressing NS induces potent immunogenicity and moreover the vaccination of the viral vector plus DNA boost induced vigorous and broad T cell responses (111, 112). In the adenoviral vector immunized chimpanzees, all challenged chimpanzees became infected but showed shorter duration of viremia

compared to unvaccinated animals (111). Some studies using this kind of vaccination are preceding clinical trials (113).

Inactivate whole virion vaccination has been authentic protocol for many virus including hepatitis A virus, Japanese encephalitis virus and rabies virus (112, 114). In HCV, the protocol has never been evaluated for the efficacy because until recently HCV had been unable to produce *in vitro*. Recently cell culture-generated HCV (HCVcc) system was developed for the first time (44). It took for eight years to develop the method to produce enough HCVcc to inoculate into mice (115). HCVcc induced NAb against HCV envelope protein in mice. These mice sera containing NAb inhibited HCV infection *in vitro* and purified IgG from the immunized mice prevented HCV infection *in vivo* in a humanized mice model (115). According to previous mice study, inactivated whole HCV particles may be promising as a potent hepatitis C vaccine. To apply the HCVcc into a clinical study, it is necessary to investigate the efficiency and side effects in non-human primates which have similar immune system to humans. Thus I tried to inoculate inactivated whole virion of HCVcc purified from mass cultivation into non-human primates for the first time.

K3-SPG is complex of K type of CpG oligodeoxynucleotides (ODNs) and schizophyllan (SPG), a soluble β -glucan derived from *Schizophyllum commune* (116). Briefly, CpG motif is a potent agonist for Toll-like receptor 9, which activates dendritic cells and B cells to produce type I IFNs and inflammatory cytokines (117, 118) and the CPG acts as an adjuvant toward both Th1-type humoral and cellular immune responses. K-type CpG ODN contains non-palindromic multiple CpG motifs, and strongly activates B cells to produce IL-6 and plasmacytoid dendritic cells to maturation but barely produces IFN- α (119, 120). K-type CpG ODN enhances the immunogenicity of

vaccines targeting infectious diseases and cancers in human clinical trials (121, 122). SPG is a drug that has been approved in Japan as an enhancer of radiotherapy in cervical carcinoma patients for the last three decades (123). It has been shown to form a complex with polydeoxyadenylic acid (dA) as a triple-helical structure (124). This novel K3-SPG forms a high order nanoparticle that can be completely solubilized and appears to act as a potent adjuvant for induction of both humoral and cellular immune responses in murine and non-human primate models (116).

Taking these situations into consideration, I sought to evaluate whether immunization of the marmosets with purified and inactivated HCVcc together with adjuvant Alum or K3-SPG could potentially induce cellular immune responses. For this purpose, I initially established a proper method to stimulate peripheral blood mononuclear cells (PBMC) and splenocytes by HCV core peptide (genotype1 or 2a) and quantitatively analyze the marmoset *IFN γ* mRNA. As hepatitis C vaccine candidates, I used HCVcc which was generated by my collaborators; Dr. Kato and Mr. Yokokawa et al. in Department of Virology II, National institute of Infectious Diseases. The HCVcc is J6/JFH-1 chimera, combination of the original JFH-1 NS2-NS5B region with the core-E2 region from another genotype 2a isolate (J6), resulted in intragenotypic chimeras with enhanced replicative fitness and production of infectious HCV particles (31, 125-127). After immunization into marmosets, I observed safety of the vaccine candidate and examined PBMC and splenocytes obtained from the vaccine-immunized marmosets for the induction of *IFN γ* mRNA by stimulation with HCV core peptides. In addition, Mr. Yokokawa examined inductions of neutralizing antibodies. This is the first report that cellular immune response was induced in HCV whole virion immunized non-human primates.

1.3. Materials and Methods

Animals

All animal studies were conducted in accordance with the protocols of experimental procedures that were approved by the Animal Welfare and Animal Care Committee of the Primate Research Institute of Kyoto University, Inuyama, Japan. A Japanese macaque (*Macaca fuscata*) and ten common marmosets (weighing 284-413 g) were used. The Japanese macaque was collected blood when euthanization as a multiple usage. Common marmosets were caged individually at $27 \pm 2^{\circ}\text{C}$ in $50 \pm 10\%$ humidity with a 12h light-dark cycle (lighting from 7:00 to 19:00). All marmosets were fed twice a day with a standard marmoset diet supplemented with fruit and mealworm. Water was given *ad libitum*. Marmoset blood were collected under anesthesia (ketamine, <10 mg/head, intramuscularly) in two weeks interval. Spleens were collected after they were euthanized by ketamine (10 mg/kg, intramuscularly), pentobarbital (50 mg/head, intraperitoneally) and then exsanguination. Blood and spleens were collected.

Cell culture

Huh7 cells (128) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) under 5% CO_2 conditions.

RNA synthesis and transfection of HCV constructs

Plasmid pJ6/JFH-1 was generated by replacing pJFH-1 structural region with that of the J6 (115).

RNA synthesis and transfection were performed as described previously (44). In

brief, plasmids were linearized with XbaI, treated with mung bean nuclease (New England Biolabs), and purified. Linearized and purified DNAs were used as templates for *in vitro* RNA synthesis using a MEGAscript T7 kit (Ambion) in accordance with the manufacturer's instructions. Synthesized RNA was treated with DNase I (Ambion), followed by purification using ISOGEN-LS (Nippon Gene). Ten micrograms of *in vitro*-synthesized RNA was used for each electroporation. (129). RNA was mixed with 400 µl of cell suspension, and the mixture was then transferred to an electroporation cuvette (Precision Universal Cuvettes). The cells were then pulsed at 260 V and 950 µF using a Gene Pulser II apparatus (Bio-Rad).

Virus production from cells transfected with full-length J6/JFH-1 chimeric virus RNA

Huh7 cells (5×10^5) were cultured in 10-cm culture dishes at 37°C under 5% CO₂. After cultivation for one day, infectious HCV clone (J6/JFH-1, genotype 2a/2a) was added at M.O.I. of 0.3 into the Huh7 and cultured for two hours. During the cultivation, the cells were shaken every 15 minutes. After two hours, the supernatant was removed, washed by phosphate buffered saline (PBS) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Sub confluent cells were passaged into 225 cm² flask (Corning) and then passaged into CellSTACK (10 chambers, Corning). The cells were washed one day after cultivation and then supernatant was harvested three days and five days after the wash. The harvested supernatant (63.6 l) was passed through 0.45-µm filter.

Concentration and purification of harvested HCVcc

Harvested supernatant containing infectious HCV particles described above were concentrated 80-fold using a hollow fiber filtration, UFP-500-C-8A (GE Healthcare) under 4-fold transmembrane pressure difference, at constant speed 1 l/min. After four times diafiltration using same amount of PBS, 250 ml PBS was passed through the ultrafiltration membrane at constant speed 0.5 l/min for five minutes and mixed with the filtrated supernatant.

Sucrose gradient ultracentrifugation

stepwise sucrose gradient (20-60%, wt/vol), 20 mM Tris-HCL (pH7.4), 150 mM NaCl, 0.05 mM EDTA (TNE Buffer) were layered into Ultra-Clear™ Centrifuge Tubes (BECKMAN COULTER) and concentrated supernatant described above (23 ml) were layered onto the stepwise sucrose gradient and centrifuged for four hours in a SW28 rotor (BECKMAN COULTER) at 28,000 rpm at 4°C. After ultracentrifugation, 12 fractions were collected. The HCV core protein levels in each fraction were determined by Lumipulse (FUJIREBIO INC.). As the peak fractions of HCV core protein (fraction 3-5) contain infectious particles the most (115), the peak fractions of HCV core protein were collected as the final product after passing through Amicon ultrafiltration membrane (Millipore) to substitute sucrose by PBS. The HCV core protein level of the final product was determined by Lumipulse (FUJIREBIO INC.). The total protein level was determined by Bradford using protein assay reagent (Bio-Rad), the refining efficiency was calculated. The final product containing HCV particle was inactivated by ultraviolet irradiation for five minutes and stocked at -80°C.

Infectivity assay of HCVcc particle

The collected samples by sucrose gradient centrifugation were serially diluted and used for inoculation of the seeded Huh7 cells, and the plates were incubated for three days at 37°C. The cells were fixed in methanol for 15 min at -20°C, and the infected foci were visualized by immunofluorescence as described previously (127). In brief, cells were blocked for one hour with BlockAce (Dainippon Sumitomo Pharma) supplemented with 0.3% Triton X-100 and then washed with PBS, followed by incubation with anti-core antibody 2H9 (10 µg/ml) in BlockAce (44). After incubation for one hour at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes) in BlockAce. The cells were then washed and examined using fluorescence microscopy (Olympus). Infectivity was quantified by counting the infected foci and expressed as numbers of focus-forming units per milliliter (FFU/ml).

Western blotting of harvested supernatant

The naive Huh7.5.1 cells that were used as a negative control and the J6/JFH-1-infected Huh7.5.1 cells that were used as a positive control were lysed using Passive Lysis Buffer (Promega), and then sonicated to lyse debris completely. The protein concentration was quantified by Bradford using Protein Assay Regent (Bio-Rad). The HCV core purified from HCV particles (10 µg protein) and cell lysate samples (10 µg protein) then were denatured by boiling, resolved on 10% sodium dodecyl sulfate polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore). The membrane was blocked with 2% skim milk of Tris-buffered saline containing Tween-20 (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% [vol/vol] Tween-20 [polyoxyethylene sorbitan monolaurate]; called TBS-T), and probed with primary antibodies against HCV

core (2H9, 1 µg/mL), E1 (B7567, 10 µg/mL, (130)), and E2 (SLT-8, 1 µg/mL; mouse IgG recognizing an epitope within amino acid residues 412-426 on E2, unpublished data) proteins in TBS-T. After several washes with TBS-T, the membrane was probed with ECL anti-mouse IgG, horseradish peroxidase (HRP) -linked whole antibody (GE Healthcare, 1:10,000 dilution) or anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, 1:5,000 dilution), washed repeatedly, and bound antibodies were detected using ECL-prime (GE Healthcare) and visualized using Image Quant LAS4000 mini (GE Healthcare).

Adjuvants

Aluminium hydroxide (Alum) was purchased from Thermo SCIENTIFIC. K3-SPG was kindly gifted from Ken J. Ishii (116). Briefly, K type of CpG ODNs were synthesized by GeneDesign (underline indicates phosphorothioate bonds): ODN (K3) (5'-ATC GAC TCT CGA GCG TTC TC-3'); K3 - 40-mer polydeoxyadenylic acid (dA 40) (5'-ATC GAC TCT CGA GCG TTC TC-40mer A-3') was purchased from Seikagaku Kogyo. K3-dA 40 (7.22 mg) was dissolved in water (3.7 ml). SPG (15 mg) was dissolved in 0.25 N NaOH (1 ml). One milliliter volume of 330 mM NaH₂PO₄ was added to the DNA solution, and then the SPG solution was added to the DNA/NaH₂PO₄ solution and kept at 4°C overnight to complete the complexation of CpG ODN and SPG. The molar ratio (M SPG: M DNA) was fixed at 0.27.

Inoculation of HCVcc particle and adjuvants into marmosets

Alum (40 mg/ml) was vortexed for several tens of seconds, K3-SPG (1.27 mg/ml) was mixed gently. Inactivated HCVcc (60 pmol/head) was mixed with Alum (250 µg/head)

or K3-SPG (90 µg/head) and added saline to make final volume 1 ml/head. The final products were divided equally for intramuscular and subcutaneous injection and inoculated into marmosets (n= 4 at twice vaccinations or n= 6 at three times vaccinations, respectively, total n= 10). The marmosets for twice vaccinations or three times vaccinations were divided into two groups; HCVcc with Alum and HCVcc with K3-SPG, respectively. The vaccination schedule was divided into two groups; twice vaccinations (week 0 and 4 after first inoculation) and three times vaccinations (week 0, 4 and 8 after first inoculation) as shown in Fig. 2. Blood was collected at once in two weeks until ten weeks after inoculation. To confirm the safety of the vaccines, body weight and clinical symptoms were checked and vaccinated animals were dissected pathologically.

Biochemical test and complete blood count

The basic biochemical test was measured by a DRI-CHEM7000 (FUJIFILM). Complete blood count was measured by a pocH-100iV (SYSMEX). The measured markers are alanine aminotransferase (ALT, U/l), aspartate aminotransferase (AST, U/l), albumin (ALB, g/ml), γ -glutamyl transpeptidase (γ GTP, U/l), alkaline phosphatase (ALP, U/l), lactate dehydrogenase (LDH, U/l), total protein (TP, g/ml), blood urea nitrogen (BUN, mg/ml), C-reactive protein (CRP, mg/ml), red blood cells (RBC, $\times 10^4/\mu\text{l}$), white blood cells (WBC, $\times 10^4/\mu\text{l}$), platelet count (PLT, $\times 10^4/\mu\text{l}$).

Isolation of PBMC, splenocytes

A healthy Japanese macaque PBMC were isolated from whole blood by Ficoll-Paque PLUS (GE Healthcare Life Sciences) and were hemolyzed by ACK lysis buffer (0.15M

NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA) at room temperature for five minutes and washed by PBS containing 1% FBS. The PBMC were divided into fresh samples and frozen samples. For frozen samples, PBMC were frozen with CELLBANKER 1 (ZENOAQ) at -80°C until cultivation. The freezing samples were thawed by incubation in 37°C water bath for one minute. Thawed samples were immediately washed once and resuspended in RPMI-1640 containing 10% FBS. Cell viability and number were checked by trypan blue exclusion method.

Marmoset spleens were collected at necropsy and splenocytes were isolated using 70 µm Cell Strainer (Falcon) and washed by RPMI-1640 containing 10% FBS. Collected splenocytes samples were hemolyzed by ACK lysis buffer (0.15M NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA) at room temperature for five minutes and washed by PBS containing 1% fetal bovine serum. Marmoset PBMC were isolated from whole blood which collected pre and post vaccination in two weeks intervals by Ficoll-Paque PLUS (GE Healthcare Life Sciences). Collected PBMC samples were hemolyzed by ACK lysis buffer (0.15M NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA) at room temperature for two minutes and washed by PBS containing 1% fetal bovine serum. The splenocytes and PBMC were frozen with CELLBANKER 1 (ZENOAQ) at -80°C until cultivation. The freezing samples were thawed by incubation in 37°C water bath for one minute. Thawed samples were immediately washed once and resuspended in RPMI-1640 containing 10% FBS. Cell viability and number were checked by trypan blue exclusion method.

Cultivation with HCV core peptides *in vitro*

To investigate the proper cultivation time and concentration of concanavalin A (ConA,

stimulator of T cells), Japanese macaque PBMC (5×10^5 cells/well) were cultured in 96 well round-bottom plate (Thermo SCIENTIFIC) at 37°C for 6, 12, 24, 36, 48 hour with the ConA (5, 50, 500 ng/well).

The marmoset splenocytes or PBMC (5×10^5 cells/well) were cultured in 96 well round-bottom plate (Thermo SCIENTIFIC) at 37°C for one hour before stimulation. The samples were stimulated by HCV core peptides (0.1, 1, 10 μ g/well); GT1 (HCV-213, genotype 1, PROSPEC), GT2a (HCV-214, genotype 2a, PROSPEC), GT1b245 (HCV-245, genotype 1b, PROSPEC), GT1bBAJ (IT-004-057Ep, genotype 1b, Immune Technology Corp) or ConA (5 μ g/ml) for six hours at 37°C under 5% CO₂. RPMI-1640 containing 10% FBS with or without ConA (5 μ g/ml) was added to the cells as a positive or negative control, respectively. After 6-hour stimulation, the splenocytes and PBMC were collected for RNA isolation.

Measurement of *IFN* γ mRNA levels

Total RNA was isolated from PBMC and splenocytes using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. Purified RNA was reverse transcribed to cDNA using ReverTra-Plus (TOYOBO). Japanese macaque and marmoset *IFN* γ mRNA expression level was quantified by real-time PCR using a THUNDERBIRD SYBER qPCR Mix (TOYOBO) on an iQ5 Multicolor real-time PCR detection system (Bio-Rad). To detect *IFN* γ , the primers cjIFN γ F [5'-CAACCAGAGCATCCAAAAGAG -3'], and cjIFN γ R [5'-AGGTGCTGGTGACAGTTCAG -3'] were used. To detect *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), the primers cjGAPDH RT_up F [5'-AAATCCCATCACCATCTTCCAG -3'] and cjGAPDH RT_up R [5'-

CTTGAGGCTGTTGTCATACTTCTC -3'] were used. The protocol of real-time PCR to detect *GAPDH* and *IFN γ* mRNA was indicated below.

95° C	1 min	1 cycle
95° C	15 sec	40 cycles
55° C	30 sec	
72° C	1 min	
55-95° C (0.5° C $\uparrow$ /cycles)	10 sec	81 cycles

The mRNA expression levels were normalized to the expression of a housekeeping gene, Japanese macaque/marmoset *GAPDH*. Fold change was represented as below mathematical formula.

$$2^{-\Delta\Delta Ct} [2^{-\{Ct (IFN\gamma, stimulation) - Ct (GAPDH, stimulation)\}} - \{Ct (IFN\gamma, negative control) - Ct (GAPDH, negative control)\}}]$$

To normalize the differences between samples, the *IFN γ* mRNA expressions in PBMC or splenocytes stimulated HCV core or ConA were divided by the *IFN γ* mRNA in PBMC or splenocytes without stimulation. To normalize the differences between individuals, the *IFN γ* mRNA expressions in splenocytes were divided by the *IFN γ* mRNA in PBMC from whole blood collected before vaccination.

Antibody titers

One microgram per milliliter of J6CF E2/FLAG recombinant protein (genotype 2a), THE2-His (genotype 1b) or 3flag-THE2 (genotype 1b) were incubated and blocked in Nunc Immuno Plate (NuncTM). Marmoset plasmas were diluted 1:100-10000 with PBS and added into the plate for 1.5 hours at room temperature. After washing, HRP-anti

monkey IgG (BETHYL) was added into the plate and incubated at room temperature for one hour. After washing, staining was measured using Peroxidase assay kit (type T, SUMITOMO BAKELITE CO., LTD.) and a plate reader (Bio-Rad or PerkinElmer) at 450 nm.

Neutralizing assay

Naïve Huh7 cells (1×10^3) were seeded into a poly-D-lysine coated 96 well plate (Corning). One hundred FFU of chimeric HCVcc (J6/JFH-1; genotype 2a, TH/JFH-1; genotype 1b, H77/JFH-1; genotype 1a and S310/JFH-1; genotype 3a) was mixed with purified marmoset antibody (30-300 $\mu\text{g/ml}$) and incubated for 30 minutes at room temperature. Naïve Huh7 cells were inoculated with the HCVcc-antibody mixtures, and incubated for six hours at 37°C in a poly-D-lysine coated 96 well plate (Corning). After incubation, the mixture was removed from cells, and 10% FBS D-MEM was added into each well, and cells were cultured for 48 hours. The cells were fixed and blocked for one hour by BlockAce (Dainippon Sumitomo Pharma) and washed by PBS. After incubation with anti-core antibody 2H9 (10 $\mu\text{g/ml}$) for one hour at room temperature, the cells were incubated with AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes) for one hour. The cells were washed then examined using fluorescence microscopy (Olympus). Infectivity was quantified by counting the infected foci and expressed as numbers of focus-forming units per milliliter (FFU/ml). % neutralization was calculated by comparing with foci in the HCVcc-PBS mixture-inoculated wells.

Statistical analyses

Statistical significance between groups was determined using the Welch's *t* test.

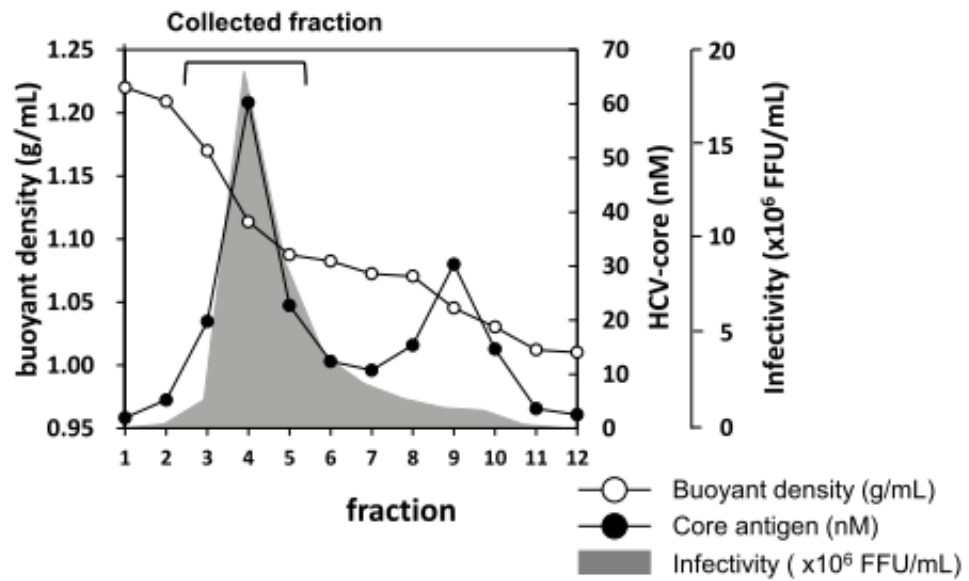
1.4. Results

Characterization of immunogenic agent, purified inactivated HCVcc particle

The HCV vaccine candidate (inactivate HCVcc) was given by Dr. Kato and Mr. Yokokawa et al. They generated and purified it as described below.

Culture supernatant (63.6 l) of Huh7 cells transfected with the full-length RNA of J6/JFH-1 chimeric virus (genotype 2a/2a) was purified and concentrated by hollow fiber filtration. Concentrated supernatant containing HCVcc particle was layered onto the stepwise sucrose gradient (20–60%, wt/vol), and ultracentrifuged. After centrifugation, 12 fractions were collected. In each fraction, buoyant density, HCV core protein levels and infectivity of HCVcc were measured (Fig 1a). As the peak fractions of HCV core protein (fraction 3-5) contain infectious particles the most, the peak fractions of HCV core protein were collected and purified using the ultrafiltration membrane to substitute sucrose by PBS. The amount of final product was 12 ml, the recovery of HCV core and total protein were 15.1%, 0.01%, respectively (Table. 1). Then, they analyzed structural proteins in the final product by western blotting. HCV E1, E2 and core proteins were detected as well as the positive control, HCV-infected Huh7 cells, but not naïve Huh7.5.1 cells (Fig. 1b). After my collaborators characterized the immunogenic agent, I inoculated it with adjuvants into marmosets, supported by Dr. Higashino.

a



b

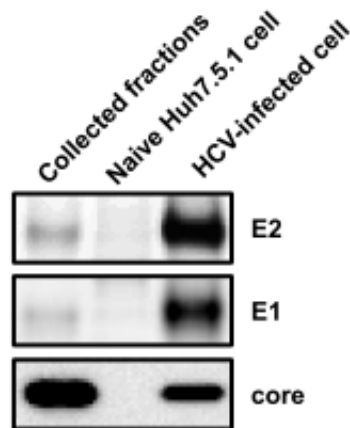


Fig. 1 Density distribution of HCV core protein and the HCV infectivity, and HCV proteins in the final product

(a) Concentrated supernatant were subjected to ultracentrifugation on a sucrose cushion. HCV core proteins in each fraction were determined by Lumipulse. Infectivity of HCVcc in each fraction were determined by titration and the results were expressed as the number of FFU per ml. (b) HCV E1, E2 and core protein in HCV core purified from HCV particles (10 μ g protein) and cell lysate samples (10 μ g protein) were confirmed by Western blotting. Samples (left) were incubated with anti-E1, E2 and core antibodies. Uninfected Huh7.5.1 cell lysates (middle) were used as a negative control, HCV-infected Huh7.5.1 cell lysates (right) were used as a positive control.

	Cell culture	Final product
Volume (l)	63.6	0.012
Recovery rate of HCV core (%)	100	15.1
Recovery rate of total protein (%)	100	0.01

Table. 1 Recovery rate of collected supernatant and final product

Safety check in the inactivated HCVcc particle and adjuvants administration into marmosets

Inactivated HCVcc (60 pmol/head) with Alum (250 µg/head) or K3-SPG (90 µg/head) were divided equally and inoculated into marmosets by intramuscularly and subcutaneously following vaccine schedule (Fig. 2). To confirm the safety of the vaccines, body weight, biochemical factors, complete blood count and clinical symptoms were checked pre and post vaccinations in two weeks intervals. The vaccinated animals were dissected pathologically. Through the vaccination, all marmosets did not show any clinical symptoms like rubor and edema (Fig. 3a, b). Both groups of marmosets inoculated with the vaccines with Alum or K3-SPG did not show any clinically changes in body weight (Fig. 3c) and the other biochemical (ALT, AST, ALB, γGTP, ALP, LDH, TP, BUN, CRP) and complete blood count (RBC, WBC, PLT) markers (table. 2-13). No significant pathological abnormality was observed in all the tissues examined, including liver, spleen, lung, heart and gastrointestinal tract (data not shown).

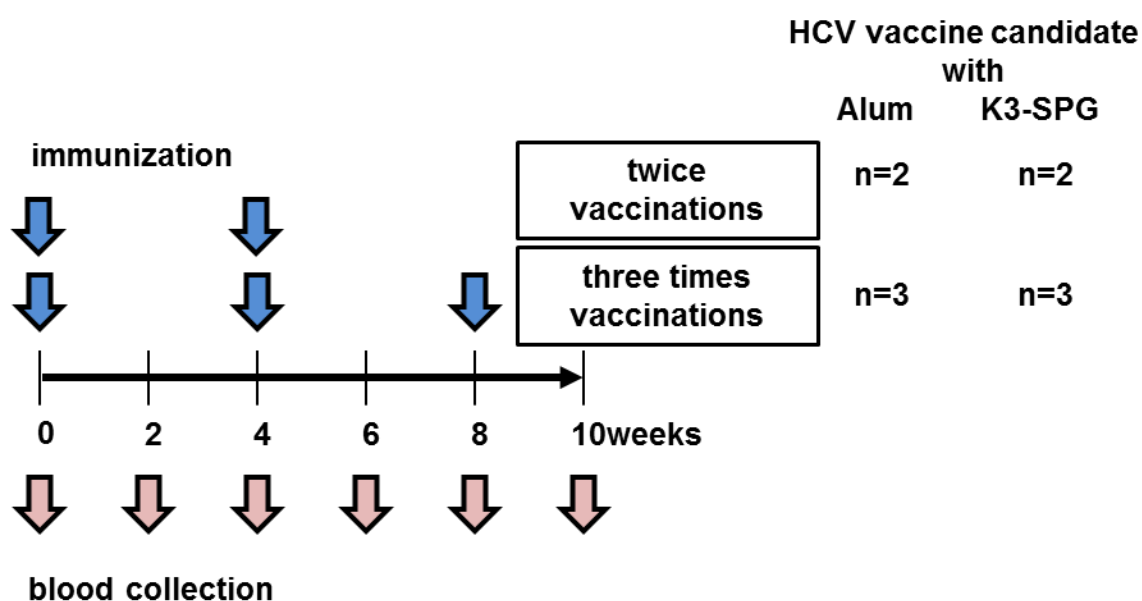


Fig. 2 Vaccine schedule

Vaccine schedule was divided into twice vaccinations (immunization 0, 4 weeks after first inoculation) and three times vaccinations (immunization 0, 4 and 8 weeks after first inoculation). Four marmosets and six marmosets were inoculated HCVcc particle with adjuvants in twice or three times vaccinations, respectively. These marmosets were divided into Alum or K3-SPG inoculated groups in each vaccination schedule.

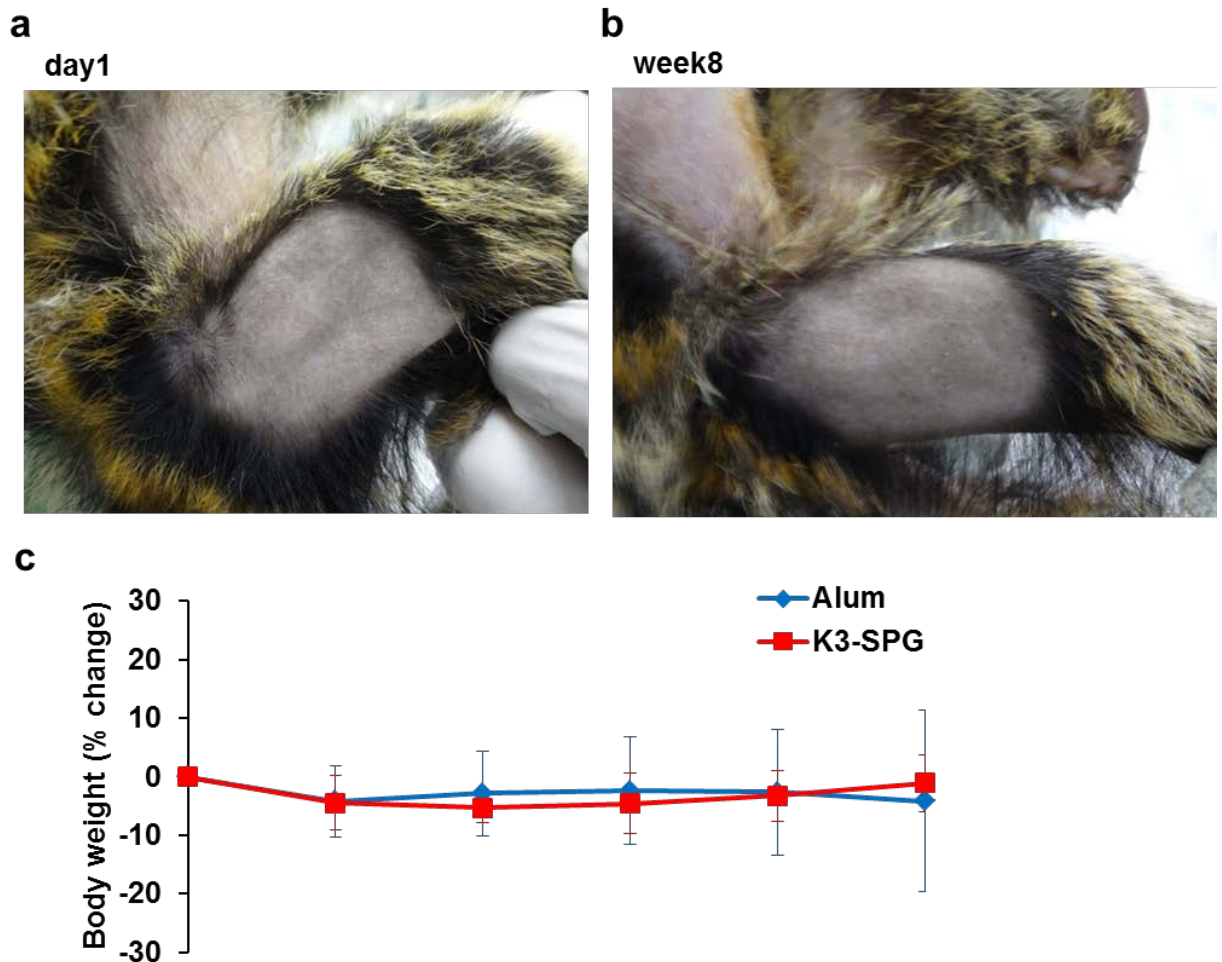


Fig. 3 Safety check

After inoculation of HCVcc particle with adjuvant, clinical symptoms were checked. (a, b) Through the vaccination, all marmosets didn't show any clinical symptoms like rubor and edema on subcutaneously infused area. (c) % changes of body weights in both Alum and K3-SPG inoculated groups were small.

ALT (U/l)	week	0	2	4	6	8	10
Alum	Cj336	7	7	10	7	5	
	Cj428	3	7	8	11	6	
	Cj476	5	4	1	1	2	3
	Cj482	9	7	6	1	2	1
	Cj478	10	4	9	3	3	1
	mean	6.80	5.80	6.80	4.60	3.60	1.67
	SD	2.86	1.64	3.56	4.34	1.82	1.15
K3-SPG	Cj405	8	15	9	14	10	
	Cj415	4	7	5	7	5	
	Cj477	2	3	2	1	2	1
	Cj483	1	6	3	1	1	1
	Cj479	10	6	1	1	2	23
	mean	5.00	7.40	4.00	4.80	4.00	8.33
	SD	3.87	4.51	3.16	5.76	3.67	12.70

Table.2 Periodical sampling data of ALT in plasma

SD means standard deviation.

AST (U/l)	week	0	2	4	6	8	10
Alum	Cj336	70	46	55	53	53	
	Cj428	72	61	59	48	59	
	Cj476	48	38	41	41	39	59
	Cj482	64	52	49	40	46	50
	Cj478	48	51	45	45	51	54
	mean	60.40	49.60	49.80	45.40	49.60	54.33
	SD	11.70	8.44	7.29	5.32	7.54	4.51
K3-SPG	Cj405	42	53	52	48	46	
	Cj415	76	45	42	45	47	
	Cj477	51	39	36	36	32	42
	Cj483	44	45	38	38	41	36
	Cj479	63	42	42	46	48	70
	mean	55.20	44.80	42.00	42.60	42.80	49.33
	SD	14.24	5.22	6.16	5.27	6.61	18.15

Table. 3

Periodical sampling data of AST in plasma

SD means standard deviation.

ALB (g/ml)	week	0	2	4	6	8	10
Alum	Cj336	5.2	5.0	4.9	5.1	5.0	
	Cj428	4.2	4.9	5.1	5.1	5.3	
	Cj476	5.8	5.5	5.6	5.5	5.6	5.4
	Cj482	5.4	5.4	5.4	5.1	4.8	4.9
	Cj478	5.4	5.3	5.0	5.2	5.1	4.8
	mean	5.20	5.22	5.20	5.20	5.16	5.03
	SD	0.60	0.26	0.29	0.17	0.30	0.32
K3-SPG	Cj405	6.0	5.5	5.1	5.2	5.0	
	Cj415	4.6	5.8	5.5	5.4	5.3	
	Cj477	3.8	4.3	4.3	4.1	4.1	4.0
	Cj483	4.9	4.7	5.0	4.6	4.4	4.5
	Cj479	5.9	5.3	5.3	5.3	5.2	5.0
	mean	5.04	5.12	5.04	4.92	4.80	4.50
	SD	0.92	0.61	0.46	0.55	0.52	0.50

Table. 4

Periodical sampling data of ALB in plasma

SD means standard deviation.

γ GTP (U/l)	week	0	2	4	6	8	10
Alum	Cj336	7	6	9	7	7	
	Cj428	5	5	4	3	4	
	Cj476	5	3	4	4	4	2
	Cj482	5	4	3	4	5	6
	Cj478	3	3	3	3	4	2
	mean	5.00	4.20	4.60	4.20	4.80	3.33
	SD	1.41	1.30	2.51	1.64	1.30	2.31
K3-SPG	Cj405	5	5	6	4	3	
	Cj415	14	6	7	5	5	
	Cj477	5	5	4	3	4	1
	Cj483	7	4	5	4	2	1
	Cj479	3	2	3	3	2	1
	mean	6.80	4.40	5.00	3.80	3.20	1.00
	SD	4.27	1.52	1.58	0.84	1.30	0.00

Table. 5

Periodical sampling data of γ GTP in plasma

SD means standard deviation.

ALP (U/l)	week	0	2	4	6	8	10
Alum	Cj336	81	47	70	86	94	
	Cj428	143	79	83	52	140	
	Cj476	61	51	59	38	48	107
	Cj482	124	89	67	80	75	174
	Cj478	52	76	44	65	94	112
	mean	92.20	68.40	64.60	64.20	90.20	131.00
	SD	39.71	18.41	14.40	19.75	33.62	37.32
K3-SPG	Cj405	70	112	77	62	61	
	Cj415	134	60	72	72	88	
	Cj477	99	87	81	106	79	130
	Cj483	105	106	60	111	105	97
	Cj479	120	49	63	67	53	75
	mean	105.60	82.80	70.60	83.60	77.20	100.67
	SD	24.11	27.71	8.96	23.07	20.86	27.68

Table. 6
Periodical sampling data of ALP in plasma
SD means standard deviation.

LDH (U/l)	week	0	2	4	6	8	10
Alum	Cj336	253	123	128	211	139	
	Cj428	155	210	147	148	163	
	Cj476	84	71	79	83	69	135
	Cj482	68	71	75	50	59	80
	Cj478	128	106	79	88	78	119
	mean	137.60	116.20	101.60	116.00	101.60	111.33
	SD	73.19	57.09	33.49	63.79	46.38	28.29
K3-SPG	Cj405	83	176	103	103	97	
	Cj415	304	84	83	80	77	
	Cj477	120	91	117	85	88	137
	Cj483	64	60	52	51	66	61
	Cj479	283	82	65	97	102	391
	mean	170.80	98.60	84.00	83.20	86.00	196.33
	SD	114.05	44.80	26.63	20.20	14.68	172.82

Table. 7
Periodical sampling data of LDH in plasma
SD means standard deviation.

TP (g/ml)	week	0	2	4	6	8	10
Alum	Cj336	4.5	3.9	4.0	4.3	4.7	
	Cj428	3.8	3.9	4.5	4.0	4.8	
	Cj476	5.0	4.5	5.0	4.6	4.7	5.7
	Cj482	4.8	4.8	4.7	4.3	4.4	5.7
	Cj478	4.0	4.5	4.3	4.5	4.7	5.2
	mean	4.42	4.32	4.50	4.34	4.66	5.53
	SD	0.51	0.40	0.38	0.23	0.15	0.29
K3-SPG	Cj405	5.1	4.8	4.0	4.4	4.6	
	Cj415	6.2	4.5	4.4	4.5	5.1	
	Cj477	3.6	3.8	3.9	3.9	3.8	4.4
	Cj483	5.2	4.7	4.7	5.0	4.7	4.8
	Cj479	5.6	4.2	4.5	4.7	4.6	5.2
	mean	5.14	4.40	4.30	4.50	4.56	4.80
	SD	0.96	0.41	0.34	0.41	0.47	0.40

Table. 8

Periodical sampling data of TP in plasma

SD means standard deviation.

BUN (mg/ml)	week	0	2	4	6	8	10
Alum	Cj336	14.1	13.2	17.6	16.5	17.0	
	Cj428	24.3	26.0	24.8	15.4	22.0	
	Cj476	36.2	19.5	16.8	16.3	13.7	20.5
	Cj482	19.1	16.2	14.0	15.8	15.7	20.3
	Cj478	14.5	19.5	17.5	16.4	23.7	20.4
	mean	21.64	18.88	18.14	16.08	18.42	20.40
	SD	9.13	4.77	4.00	0.47	4.25	0.10
K3-SPG	Cj405	12.9	20.7	13.2	12.5	10.9	
	Cj415	14.2	11.1	17.5	15.2	14.7	
	Cj477	16.0	13.8	17.5	17.8	19.5	19.7
	Cj483	17.6	16.1	17.2	26.6	23.1	17.1
	Cj479	22.9	14.6	12.3	15.4	14.0	17.8
	mean	16.72	15.26	15.54	17.50	16.44	18.20
	SD	3.89	3.54	2.57	5.42	4.83	1.35

Table. 9

Periodical sampling data of BUN in plasma

SD means standard deviation.

CRP (mg/ml)	week	0	2	4	6	8	10
Alum	Cj336	0.3	0.3	0.3	0.3	0.3	
	Cj428	0.3	0.3	0.3	0.3	0.3	
	Cj476	0.3	0.3	0.3	0.3	0.3	0.3
	Cj482	0.3	0.3	0.3	0.3	0.3	0.3
	Cj478	0.3	0.3	0.3	0.3	0.3	0.3
	mean	0.3	0.3	0.3	0.3	0.3	0.3
	SD	0	0	0	0	0	0
K3-SPG	Cj405	0.3	0.3	0.3	0.3	0.3	
	Cj415	0.3	0.3	0.3	0.3	0.3	
	Cj477	0.3	0.3	0.3	0.3	0.3	0.3
	Cj483	0.3	0.3	0.3	0.3	0.3	0.3
	Cj479	0.3	0.3	0.3	0.3	0.3	0.3
	mean	0.3	0.3	0.3	0.3	0.3	0.3
	SD	0	0	0	0	0	0

Table. 10
Periodical sampling data of CRP in plasma
SD means standard deviation.

RBC ($\times 10^4/\mu\text{l}$)	week	0	2	4	6	8	10
Alum	Cj336	617	584	683	688	668	
	Cj428	595	533	615	570	565	
	Cj476	409	446	468	438	452	480
	Cj482	521	486	432	423	455	484
	Cj478	447	505	488	506	514	503
	mean	517.80	510.80	537.20	525.00	530.80	489.00
	SD	90.36	51.72	106.76	108.31	89.76	12.29
K3-SPG	Cj405	680	733	692	668	636	
	Cj415	613	514	576	574	557	
	Cj477	444	416	438	428	408	445
	Cj483	450	441	426	415	397	443
	Cj479	590	506	555	571	553	570
	mean	555.40	522.00	537.40	531.20	510.20	486.00
	SD	104.35	125.14	109.53	107.57	103.81	72.75

Table. 11
Periodical sampling data of RBC in whole blood
SD means standard deviation.

WBC ($\times 10^4/\mu\text{l}$)	week	0	2	4	6	8	10
Alum	Cj336	55	25	39	41	36	
	Cj428	71	15	28	24	22	
	Cj476	36	25	39	31	34	22
	Cj482	46	52	54	40	64	43
	Cj478	25	26	21	24	25	23
	mean	46.60	28.60	36.20	32.00	36.20	29.33
	SD	17.64	13.83	12.56	8.28	16.62	11.85
K3-SPG	Cj405	55	49	68	56	31	
	Cj415	45	31	26	33	27	
	Cj477	29	32	33	39	39	19
	Cj483	37	32	35	39	88	40
	Cj479	48	34	39	47	47	52
	mean	42.80	35.60	40.20	42.80	46.40	37.00
	SD	10.06	7.57	16.24	8.90	24.49	16.70

Table. 12

Periodical sampling data of WBC in whole blood

SD means standard deviation.

PLT ($\times 10^4/\mu\text{l}$)	week	0	2	4	6	8	10
Alum	Cj336	53.4	63.8	61.1	61.4	55.2	
	Cj428	49.0	47.9	55.4	49.4	50.6	
	Cj476	85.9	73.0	66.3	67.1	71.1	71.0
	Cj482	61.2	59.3	58.5	45.5	55.1	65.5
	Cj478	68.2	84.4	72.6	78.5	83.2	101.4
	mean	63.54	65.68	62.78	60.38	63.04	79.30
	SD	14.50	13.82	6.79	13.38	13.70	19.34
K3-SPG	Cj405	72.1	73.6	69.6	55.5	57.4	
	Cj415	55.2	57.7	53.5	54.2	62.5	
	Cj477	92.5	86.9	90.3	92.3	77.2	97.4
	Cj483	71.8	63.8	63.8	63.9	59.4	80.0
	Cj479	81.5	70.4	68.2	64.4	62.9	76.7
	mean	74.62	70.48	69.08	66.06	63.88	84.70
	SD	13.77	11.04	13.43	15.40	7.78	11.12

Table. 13

Periodical sampling data of PLT in whole blood

SD means standard deviation.

Establishment of method to quantitatively measure *IFN* γ mRNA in PBMC and splenocytes

To investigate the proper cultivation time and concentration of ConA as positive stimulation controls, I used Japanese macaque PBMC because lots amount of cells are need to establish cultivation methods and the number of marmoset PBMC collected in one blood collection is not enough but that of Japanese macaque can be enough. Japanese macaque fresh PBMC (5×10^5 cells/well) were cultured with or without ConA (5, 50, 500 ng/well) at 37°C for 6, 12, 24, 36, 48 hours. After cultivation, total RNA was extracted from PBMC and then the RNA was reverse transcribed to cDNA. Japanese macaque *GAPDH* and *IFN* γ mRNA expression levels were quantified by real-time PCR using primer sets and protocol as indicated in 1.3. Materials and Methods. The protocols for measuring *GAPDH* and *IFN* γ mRNA expression levels were established by confirming that PCR efficiency was 90-105% and slope was above 0.98. *IFN* γ mRNA expression levels were calculated by $2^{-\Delta\Delta CT}$ method as indicated in 1.3. Materials and Methods. To normalize the differences between samples, the *IFN* γ mRNA expressions in PBMC stimulated with ConA were divided by the *IFN* γ mRNA in PBMC without stimulation. *IFN* γ mRNA expression induced by ConA was increased in a dose dependent manner and the level was highest in a condition of 6-hour cultivation (Fig. 4). Taking this result and another report into consideration (131), following experiments were proceeded in the condition of 6-hour cultivation with 5 μ g/ml (500ng/well) of ConA.

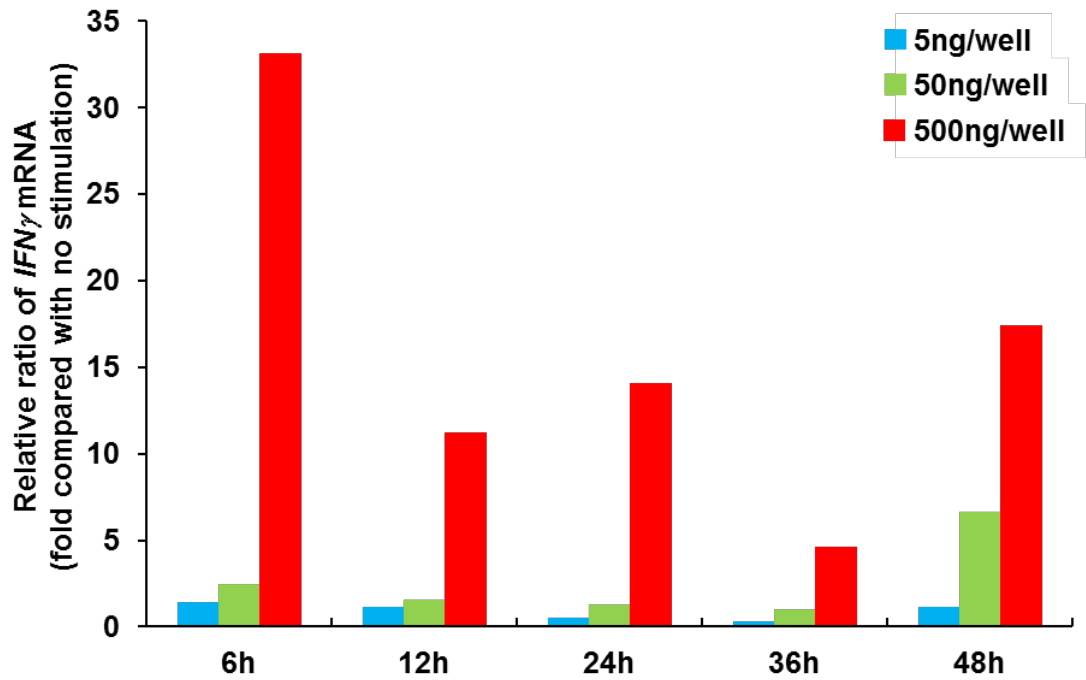


Fig. 4 Establishment of method of cultivation time and proper concentration of ConA and comparative analysis of expression of *IFN* γ mRNA in fresh and frozen PBMC

Japanese macaque fresh PBMC (5×10^5 cells/well) were cultured with or without ConA (5, 50, 500 ng/well) at 37°C for 6, 12, 24, 36, 48 hours. After cultivation, total RNA was extracted and reverse transcribed to cDNA. Japanese macaque *GAPDH* and *IFN* γ mRNA expression levels were quantified by real-time PCR. The expressions of *IFN* γ mRNA were normalized by $2^{-\Delta\Delta CT}$ method.

Selection of HCV core peptides for stimulating PBMC and splenocytes in marmosets immunized with HCVcc with adjuvants

It is known that stimulation of the common antigen with the vaccine antigen activates memory cells, and *IFN* γ mRNA in PBMC or splenocytes was induced. Thus I examined four kinds of HCV core peptides (GT2a, GT1, GT1b245, GT1bBAJ) by using frozen PBMC in marmosets immunized by HCVcc (genotype 2a) with Alum as pilot study and in naïve marmosets. Some peptides may contain toxic agents like endotoxin and may increase baseline of cytokine expression. I selected the stimulation peptides for marmosets PBMC and splenocytes which were immunized with HCVcc and Alum or K3-SPG according to this result. The thawed and resuspended PBMC (5×10^5 cells/well) were cultured for one hour without stimulation and then cultured for six hours with four kinds of HCV core peptides at different concentration (0.1, 1, 10 μ g/well) at 37°C, respectively. The expressions of *IFN* γ mRNA in PBMC from a marmoset immunized with HCVcc with Alum increased dose-dependently by co-culture with GT2a which genotype was same as HCVcc. On the other hand, the expressions of *IFN* γ mRNA in PBMC obtained before immunization were low and did not increase even by highest dose of GT2a (Fig. 5a). The expressions of *IFN* γ mRNA in PBMC obtained after immunization and from a naïve marmoset did not increase by co-culturing with GT1. The HCV core peptide (GT1) is composed of HCV core genotype 1 which is different genotype with HCVcc (genotype 2a, Fig. 5b). Notably, both of GT1b245 and GT1bBAJ increased the expressions of *IFN* γ mRNA in PBMC from naïve marmosets (Fig. 5c, d). It is possible that toxic agents contained in these peptides and increased baseline, thus co-culturing with high dose of the peptides induced apoptosis in PBMC. These results

showed that GT2a (1 μ g/well) and GT1 (1 μ g/well) were appropriate peptides for following experiments.

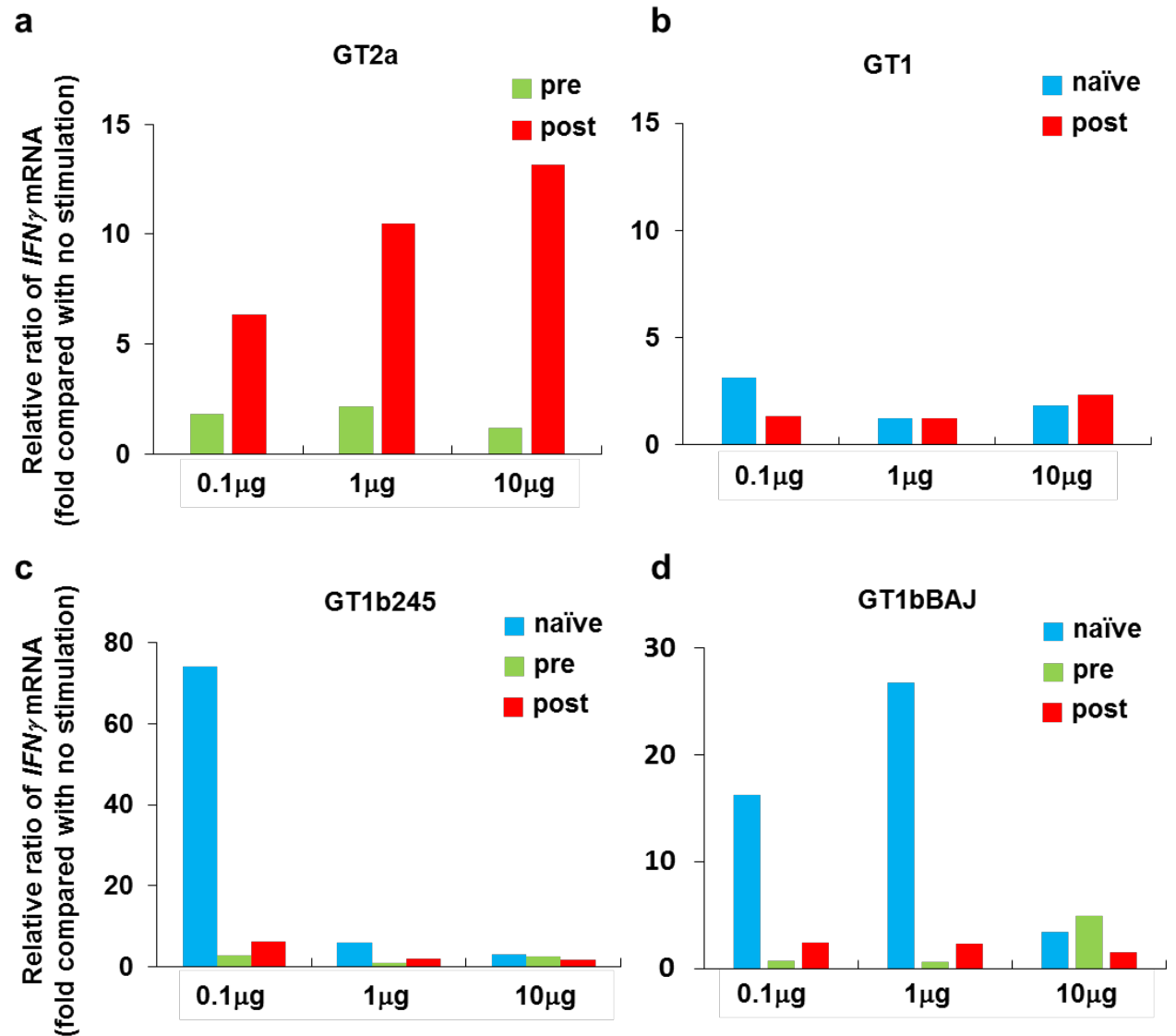


Fig. 5 Expressions of *IFNγ* mRNA in PBMC stimulated by HCV core peptides

Frozen PBMC in marmosets pre and post immunized by HCVcc with Alum as pilot study and in naïve marmosets were cultured with four kinds of HCV core peptides (GT2a, GT1, GT1b245, GT1bBAJ) at several concentration (0.1, 1, 10 μg/well), respectively. The expressions of *GAPDH* and *IFNγ* mRNA were quantified by real-time PCR. The normalized *IFNγ* mRNA expression levels were indicated. (a) PBMC in a marmoset pre or post immunized were cultured with GT2a, (b) PBMC in a naïve marmoset or a marmoset post immunized were cultured with GT1, (c) PBMC in a naïve marmoset or a marmoset pre or post immunized were cultured with GT1b245, (d) PBMC in a naïve marmoset or a marmoset pre or post immunized were cultured with GT1bBAJ.

***IFN* γ mRNA expression levels were increased in splenocytes of marmosets inoculated HCVcc with K3-SPG**

Taking the results from pilot studies into consideration, frozen PBMC collected before vaccination trial and frozen splenocytes collected after vaccination trial were co-cultured with GT 1 or GT 2a, respectively for six hours after pre-cultivation for one hour. Normalized *IFN* γ mRNA expressions in splenocytes treated with HCV core peptides (GT2a) were increased in marmosets inoculated with HCVcc and K3-SPG (Fig. 6). Especially marmosets injected with HCVcc three times induced greater *IFN* γ mRNA expressions as compared with marmosets injected with HCVcc twice. In marmosets inoculated with HCVcc and Alum three times, *IFN* γ mRNA expressions were scarcely increased. These results suggest that novel combination of inactivated HCVcc and K3-SPG induced effective cellular immune responses against HCV antigen. This candidate may be promising as an effective hepatitis C vaccine without any obvious side effects.

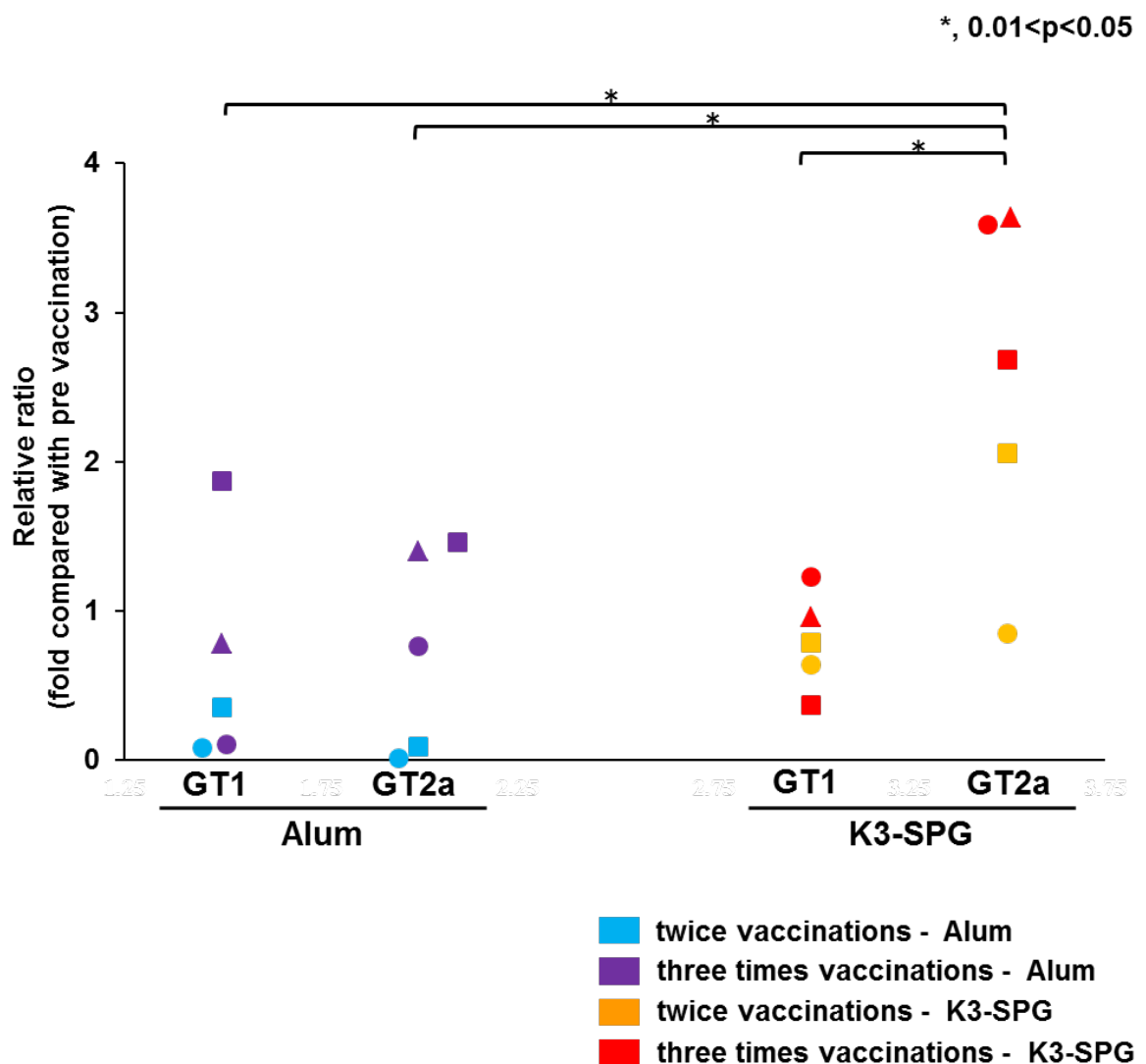
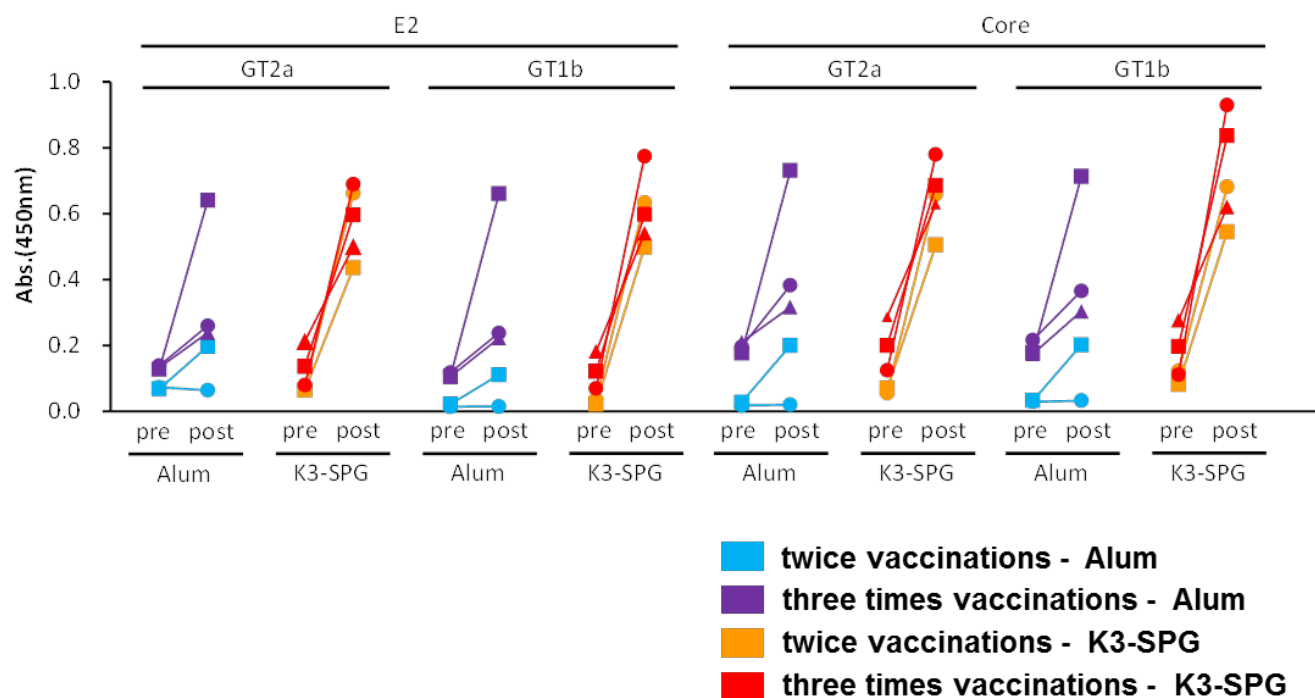


Fig. 6 *IFN*γ mRNA expression in splenocytes compared to pre-vaccinated PBMC

Isolated PBMC (pre vaccination) and splenocytes (eight weeks or ten weeks after first vaccination) were cultured with HCV core peptides (genotype 1 or genotype 2a). *IFN*γ mRNA expressions were measured by real-time PCR and normalized to the expression of a housekeeping gene (GAPDH). Fold change was represented as $2^{-\Delta\Delta C_t}$ method. To normalize the differences between individuals, the *IFN*γ mRNA expressions in splenocytes were normalized by *IFN*γ mRNA in pre-vaccinated PBMC. *IFN*γ mRNA expressions in marmosets injected HCVcc with Alum twice or three times are indicated as blue or purple. *IFN*γ mRNA expressions in marmosets injected HCVcc with K3-SPG twice or three times are indicated as orange or red.

HCVcc vaccine induced HCV core and E2 specific antibodies in marmosets

In addition, Mr. Yokokawa investigated the induction of humoral immune responses against the HCVcc with adjuvants. To investigate the induction of HCV-specific antibodies, HCV-specific antibody titers in plasmas were evaluated using EIA. Antibodies against HCV E2 (genotype 2a) were increased after vaccination especially in marmoset inoculated HCVcc with K3-SPG (Supplementary Fig. 2). The HCVcc with K3-SPG induced antibodies against cross-genotypic E2 (genotype 1b). In addition, antibodies against HCV core (genotype 2a and 1b) were increased after vaccination in marmosets inoculated HCVcc with K3-SPG. Only one marmoset inoculated HCVcc with Alum induced antibodies against HCV E2 and core (genotype 2a and 1b) significantly.

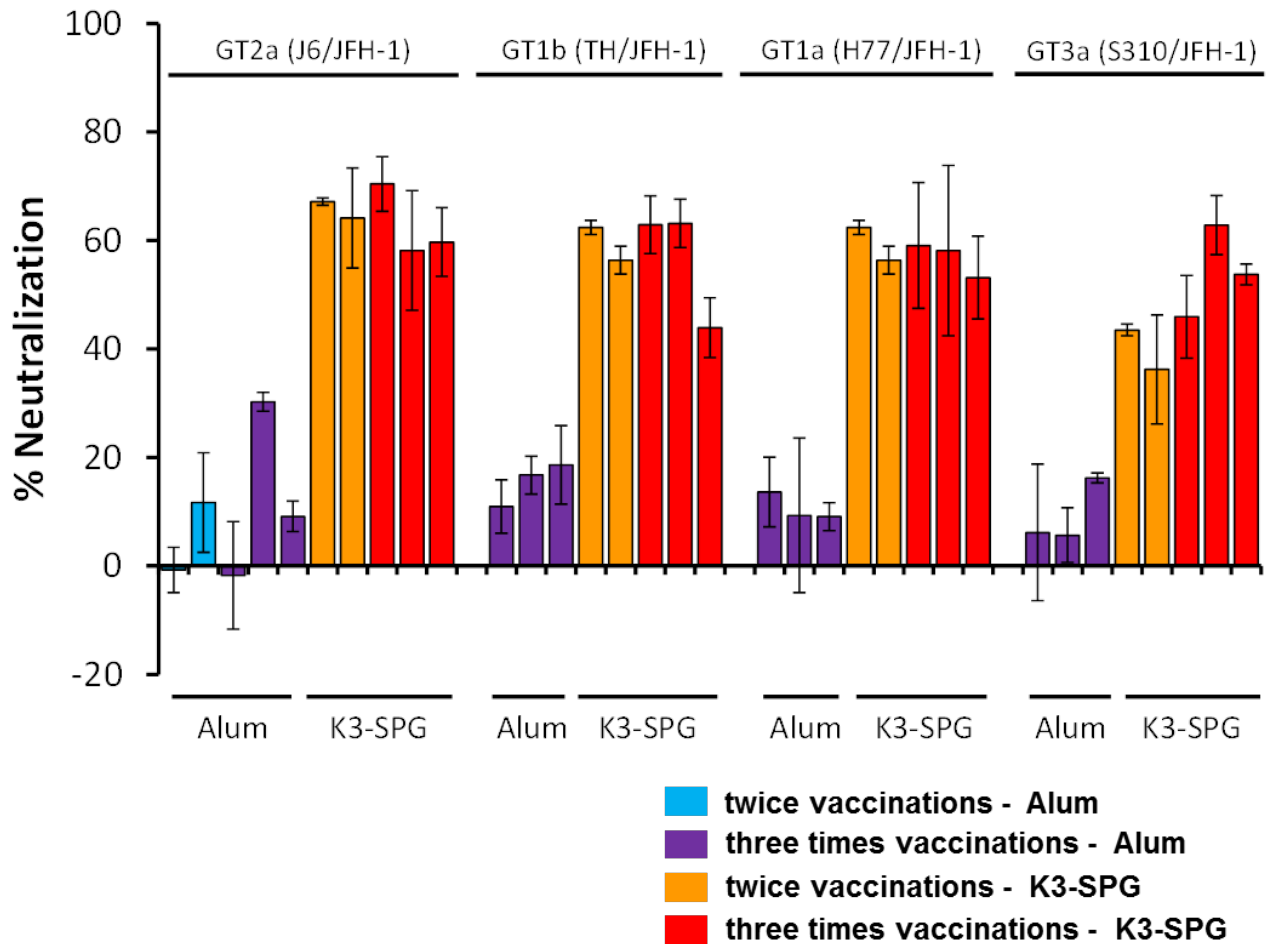


Supplementary Fig. 2 Antibody titers against HCV E2 and core (genotype 2a and 1b) before and post vaccinations

Antibody titers against HCV E2 and core in plasmas were evaluated using EIA. Antibodies in marmosets injected HCVcc with Alum twice or three times are indicated as blue or purple. Antibodies in marmosets injected HCVcc with K3-SPG twice or three times are indicated as orange or red. Antigens were used E2 and core of same genotype with HCVcc (genotype 2a) and cross-genotypic E2 (genotype 1b).

Cross-genotypic NAb were induced in marmosets inoculated HCVcc with K3-SPG

IgG in plasmas were purified and mixed with chimeric HCVcc (J6/JFH-1; genotype 2a, TH/JFH-1; genotype 1b, H77/JFH-1; genotype 1a and S310/JFH-1; genotype 3a). After cultivating Huh7 cells in 96 well plates, the mixtures were added and cultured for six hours. After cultivating, neutralization activity was evaluated by comparing with focus number in the PBS-treated HCVcc infected wells. The purified IgG in marmosets inoculated HCVcc with K3-SPG has potent neutralizing activity against J6/JFH-1 which was same genotype with HCVcc vaccine (genotype 2a, Supplementary Fig. 3). Also the induced antibodies in marmosets inoculated HCVcc with K3-SPG have neutralizing activity against TH/JFH-1, H77/JFH-1 and S310/JFH-1 which have different genotypes with HCVcc vaccine (genotype 1b, 1a and 3a). The % neutralization was not different between twice injection and three times vaccinations.



Supplementary Fig. 3 NAb against cross-genotypic HCV

IgG in plasma was purified and mixed with chimeric HCVcc (J6/JFH-1; genotype 2a, TH/JFH-1; genotype 1b, H77/JFH-1; genotype 1a and S310/JFH-1; genotype 3a). After cultivating Huh7 cells in 96 well plates, the mixtures were added and cultured for six hours. After cultivating, neutralization activity was evaluated by comparing with focus number in the PBS-treated HCVcc infected wells. NAb in marmosets injected HCVcc with Alum twice or three times are indicated as blue or purple. NAb in marmosets injected HCVcc with K3-SPG twice or three times are indicated as orange or red.

1.5. Discussion

In this study, I established the methodology to measure *IFN γ* mRNA expression of PBMC and splenocytes in immunized marmosets. Then I inoculated the inactivated HCVcc, together with either of two kinds of adjuvants into common marmosets and assessed safety of the candidates. I found no side effects against immunization with the vaccine candidates. To investigate the induction of cellular immune responses, I co-cultured PBMC/splenocytes with HCV core peptides and found that increased expressions of *IFN γ* mRNA in marmosets immunized by HCVcc with K3-SPG. The results suggest that the combination of HCVcc and K3-SPG worked as the potent and safe vaccine of HCV in marmosets. In addition, the data investigated by Mr. Yokokawa suggest that K3-SPG is a potent adjuvant for induction of cross-genotypic NAb and production of *IFN γ* mRNA. This hepatitis C vaccine candidate (HCVcc) with K3-SPG has a possibility to become an efficient hepatitis C vaccine which induces humoral and cellular immune responses. Furthermore, this vaccine candidate induced genotype 1b-NAb, indicating that this vaccine candidate might be useful to Japanese patients because 70% of them are infected genotype 1b-HCV in Japan (39).

Expressions of *IFN γ* mRNA in PBMC obtained two weeks interval did not increase. Since the numbers of PBMC were not enough, the expressions of *IFN γ* mRNA significantly varied in individuals and the collected time points. On the other hand, the numbers of splenocytes collected on euthanization were abundant, the data were reliable. In addition, it might be also because frozen PBMC were used. I compared the expression of *IFN γ* mRNA in fresh PBMC with that in frozen PBMC. Fresh Japanese macaque PBMC (5×10^5 cells/well) were cultured with or without ConA (5 μ g/ml) at

37°C for six hours. Frozen Japanese macaque PBMC were thawed by incubation at 37°C for one minute and were resuspended at 5×10^5 cells/well. The cells were cultured for one hour without stimulation and then cultured for six hours with ConA (5 µg/ml) at 37°C, as previously described with some modifications (132-134). The expression of *IFN γ* mRNA in frozen PBMC (3.8 fold compared with no stimulation) decreased significantly as compared to fresh PBMC (33.2 fold compared with no stimulation). I used frozen samples in the following experiments in order to minimize the dispersion of culture condition and other experimental schedule. Now I'm in progress to measure production of IFN γ protein in splenocytes.

In previous hepatitis C vaccine studies using chimpanzees, some kinds of hepatitis C vaccines are evaluated and they brought important insights. HCV-LPs resemble the putative HCV virions and contained HCV core, E1, E2 which were produced in insect cells. In the chimpanzees immunized with the HCV-LPs, HCV-specific cellular immune response was enhanced (135). However upon challenge with infectious HCV inoculum, viremia could not be prevented in some chimpanzees, although increase of T cell responses coincided with the fall in HCV RNA levels. In HCV (genotype 1a) recombinant E1, E2 protein-immunized chimpanzees, cross-genotypic NAb against HCV were detected. The vaccinated chimpanzees protected following HCV (genotype 1a) challenge (110). These chimpanzees developed cross-genotypic NAb after the second vaccination. But cellular immune response was not investigated. In DNA vaccine encoding cell-surface HCV E2 glycoprotein immunized chimpanzees, anti-E2 antibody was induced but could not prevent acute infection, although anti-E2 specific CD4⁺ T cell response was detected (136). As the treatment of chronic infection, anti-PD-1 antibody was inoculated chimpanzees with persistent HCV infection (137). A

significant reduction in HCV viremia was observed in one of three treated animals without apparent hepatocellular injury. But viremia rebounded when antibody treatment was discontinued. These vaccine candidates have reported that the effectiveness of vaccine immunity in mice or rhesus macaques or *in vitro*. Nevertheless, it is difficult to show prophylactic and therapeutic effects in chimpanzees which are the appropriate model for vaccine study. In addition, chimpanzee research was banned in most countries due to ethical concerns by 2013 (31).

To apply the established hepatitis C vaccine candidate (HCVcc) with K3-SPG in clinical trials for further step, it is necessary to assess whether this candidate has prophylactic/therapeutic effects or not. But an appropriate animal model is not available. In order to overcome this obstacle, I established a chimera composed of HCV and GB virus B (GBV-B) and evaluated the replication efficiency in primary marmoset hepatocytes and a red-handed tamarin (*Saguinus midas*) to develop a novel animal model in 2nd chapter.

Chapter 2

Construction and characterization of a novel hepatitis C virus/GB virus B chimera for evaluating prophylactic and therapeutic effects of the hepatitis C vaccine candidates

2.1. Abstract

The development of effective Hepatitis C virus (HCV) vaccines is essential for the prevention of further HCV dissemination, especially in the developing countries. A number of preclinical and clinical studies of HCV vaccine candidates are in progress. However, it usually needs so many years to evaluate the prophylactic and therapeutic efficacies of the vaccines in clinical trials. The animal of which immunity is comparable to human and also permissive for HCV infection is only chimpanzee, while no more available for invasive research. I thus sought to establish feasible and immunocompetent surrogate animal model of HCV infection, which would greatly help accelerate evaluating the prophylactic and therapeutic efficacies of newly developing HCV vaccine candidates. In order to circumvent narrow host range of HCV, an HCV genotype 1b-based chimeric clone carrying E1, E2 and p6 regions from GB virus B (GBV-B), which is closely related to HCV, was generated. The chimera between HCV and GBV-B, named HCV/G, replicated more efficiently as compared with the HCV clone in primary marmoset hepatocytes, and was implied to form viral particle. Furthermore, it was found that the chimera persistently replicated in a red-handed tamarin (*Saguinus midas*) for more than two years after intrahepatic inoculation of chimeric RNA. The viral RNA loads in plasma were relatively low (<200 copies/ml) but were detectable intermittently during the observation period. Of note, the chimeric RNA was found in the pellet fraction obtained by ultracentrifugation of the plasma at 73 weeks, indicating production of the chimeric virus. My results will help establish the novel non-human primate model for HCV infection on the basis of the HCV/G chimera in the major framework of HCV genome. This model will be able to evaluate hepatitis C

vaccines which induce cellular immune responses against HCV core or nonstructural proteins.

2.2. Introduction

Recent estimates of chronic Hepatitis C disease burden show an increase in seroprevalence from 2.3% to 2.8% and >122 million to >185 million infections worldwide between 1990 and 2005, the prevalence being particularly high in Central and East Asia and North Africa and the middle East (36, 138). Since the approval in 1992 of interferon α (IFN α) treatment for Hepatitis C virus (HCV), pegylated IFN α and ribavirin treatment have been the mainstay of therapy for more than 25 years and still one of the standard therapies (102, 103). Recently, IFN-free treatments using all-oral regimen of direct-acting antiviral (DAA) agents with a highly sustained virological response (SVR) rate have been approved (107, 139). The DAA therapy is extremely promising but very expensive and may therefore not be affordable in developing countries. To control further dissemination of HCV infection, it is thus essential to develop inexpensive vaccines.

The neutralizing antibodies (NAb) against HCV have been shown to be crucial for controlling the viral replication and disease progression (140-142). In particular, rapid induction of broad NAb in the early stage of HCV infection is key to the control of HCV infection. An HCV vaccine that induces NAb in chimpanzees is reportedly promising (110, 143, 144). On the other hand, cytotoxic T lymphocytes (CTL) responses also play a crucial role in viral control (93, 111, 145). Of note, it has been proposed that CTL targeting nonstructural (NS) proteins have functional significance (146-148).

HCV exhibits a narrow host range, only humans and chimpanzees being permissive for HCV infection in the presence of physiological immune responses. Thus,

chimpanzees have provided much critical information in terms of vaccine feasibility and efficacy (109, 111, 149). However, the use of chimpanzees for invasive experiments including evaluation for the prophylactic and therapeutic efficacy of vaccine candidates against HCV challenge is generally prohibited because of ethical concern for this endangered species and the high cost of rearing these animals and maintaining suitable facilities. To achieve breakthrough in this issue, a chimeric virus between HCV and GB virus B (GBV-B) has been recently featured. GBV-B is closely related to HCV, with 25–30% homology at the amino acid level. Experimental infection with it induces acute and chronic hepatitis in small New World monkeys (NWMs) such as red-handed tamarins (*Saguinus midas*), common marmosets (*Callithrix jacchus*) and owl monkeys (*Aotus trivirgatus*) as described in General Introduction “Virology of GB virus B (GBV-B)” (58, 64-69). Previous reports have shown that the chimeras, in which short pieces of HCV 5’ untranslated region (UTR), hyper variable region or p7 sequences have been replaced by corresponding GBV-B regions, have been constructed; however, their replication in NWMs *in vivo* has been highly restricted (150-154). It is possible that HCV E1E2 may not interact with orthologs in NWMs as HCV receptors, resulting in resistance of NWMs to HCV infection.

Taking these findings into consideration, I sought to construct a novel replication-competent HCV-based chimera in NWMs to establish an immune-competent surrogate model that would be capable of evaluating the efficacy of CTL-inducible vaccines targeting NS proteins.

2.3. Materials and Methods

Construction of Chimeric Genomes

The pTPF-1 HCV genotype 1b infectious clone was constructed from a patient with fulminant hepatitis C (DDBJ/EMBL/GenBank accession number LC011928, Ken-ichi Mori et al., personal communication.). The TPF1 virus generated by transfection of the viral genomic RNA is infectious in Huh7 cells. pTPF1-dGDD is a mutant defective of RNA-dependent RNA polymerase (RdRp) activity (155). The infectious molecular clone of GBV-B, pGBB, was kindly provided by Dr. Jens Bukh (NIH, Bethesda, MA, USA) (156). The HCV and GBV-B chimeric viral clone pHCV/G was constructed by swapping C-terminus of core region corresponding signal peptide sequence, E1, E2 (804-2573 nucleotides from the 5'UTR) of HCV TPF1 genome for their counterpart elements of GBV-B core signal peptide sequence, E1, E2 and p6 (815-2446 nucleotides). Restriction Enzyme *SplI* and *BbvCI* were used in these junction sites. The chimeric clone was investigated with Sanger sequences to confirm the full length of the viral genome.

***In Vitro* RNA Transcription**

XhoI-linearized plasmids were transcribed *in vitro* by T7 RNA polymerase with a MEGAscript T7 kit (Ambion). The synthesized RNAs were purified according to the manufacturer's instructions.

***In vitro* replication analyses of HCV/G**

Huh7, a human hepatocyte-derived cell line, provided by Dr. Tetsuro Suzuki (National

Institute of Infectious Diseases, Tokyo, Japan) (128) and primary marmoset hepatocytes (Biopredic international) were used for testing replication kinetics of HCV/G. Three microgram of synthesized RNAs derived from pHCV/G, pTPF1 and pTPF1-dGDD were transfected into 5×10^4 Huh7 cells or 1×10^5 primary marmoset hepatocytes according to the manufacturer's instructions (DMRIE-C; Invitrogen). The transfected cells and the culture supernatants were harvested daily after transfection. The replication of these chimeric viral RNAs was measured by sandwich enzyme immunoassay (EIA) of HCV core antigen as described previously (157, 158).

Sucrose density gradient analysis of HCV/G core and RNA

Culture medium of primary marmoset hepatocytes three and four days after transfection of HCV/G RNA was harvested for sucrose density gradient analysis. The culture supernatants were cleared by low-speed centrifugation, passed through a 0.45- μ m filter, and concentrated 30-fold using an Amicon Ultra-15 device (Molecular cut-off: 1×10^5 Da; Millipore). The concentrated supernatants with or without NP-40 pretreatment were layered onto a stepwise sucrose gradient (10–60%, wt/vol) and centrifuged for 16h in a SW50.1 rotor (BECKMAN COULTER) at 40,000 rpm with RNase A at 4°C. After centrifugation, 20 fractions were collected. The HCV core antigen levels in each fraction were determined by sandwich EIA as described above. Viral RNA in culture supernatants was purified using a QIAamp Viral RNA Mini kit (QIAGEN) and quantified by real-time PCR using a QuantiTect One-Step RT-PCR kit (QIAGEN). To detect the HCV/G 5'UTR region, primer pairs chiba-S [5'-TAGTGGTCTGCGGAACCGGT -3'] and chiba-AS [5'-TGCACGGTCTACGAGACCT -3'] were used as described previously (158).

Animals

Two adult red-handed tamarins (weighing 426-572 g) were used in this study. The tamarins were reared at the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan. These animal experiments were conducted in accordance with the protocols of experimental procedures that have been approved by the Animal Welfare and Animal Care Committees of the Primate Research Institute of Kyoto University and National Institute of Biomedical Innovation, Health and Nutrition, Japan. All animals were caged individually at $27 \pm 2^{\circ}\text{C}$ in $50 \pm 10\%$ humidity with a 12h light-dark cycle (lighting from 7:00 to 19:00). All animals were fed twice a day with a standard tamarin diet supplemented with fruit and mealworm. Water was given *ad libitum*. Blood of monkeys was collected under anesthesia (ketamine, <10 mg/head, intramuscularly).

Inoculation of GBV-B and HCV/G genome RNAs

The GBV-B infectious serum obtained from a tamarin was inoculated into a tamarin (3.3×10^7 copies/head) by intravenous injection. Four hundred microgram of HCV/G RNA was inoculated into a tamarin intrahepatically. Blood samples were periodically collected from the femoral vein of each animal under anesthesia (ketamine, <10 mg/head, intramuscularly). Plasma and PBMC were evaluated for amounts of viral genomic RNA and alanine aminotransferase (ALT).

Biochemical Test

The basic biochemical test regarding alanine aminotransferase (ALT) level in plasmas

of the tamarins was measured by a DRI-CHEM7000 (FUJIFILM).

Detection and Quantification of Viral RNA

Viral RNA in plasma and PBMC samples was purified using a QIAamp Viral RNA Mini kit (QIAGEN). Two hundred microliters of plasma was concentrated into 50 µl RNA extracts. PBMC were isolated and concentrated into 50 µl RNA as described previously (70). One microliter of RNA extract was quantified by real-time PCR using a TaqMan RNA-to-Ct 1-Step Kit (TaqMan). To detect GBV-B 5'UTR region, the primers GBV-B 5'UTR primer F90 [5'- TGCAGAAGGGTGGAGCCAACCACTTAGTA -3'] and R300 [5'- CGTCCTTGGTAGTGACCGGT -3'] were used. To detect HCV/G 5'UTR region, the primer chiba-S and chiba-AS were used as described above. For quantification of GBV-B RNA, viral copies of the standard were calculated using concentration of transcribed GBV-B RNA and number of GBV-B moles. The minimum copies detected in real-time PCR using serial diluted standard were 250 copies/ml GBV-B RNA. Regarding quantification of HCV/G RNA copies, 50 µl of the RNA extracts was aliquoted into 50 tubes (1 µl in each) and real-time PCR performed on all the tubes. Summing the quantitation results for each tube increased the threshold of RNA detection 50-fold.

Sequencing of Chimeric Genome

Viral RNA was isolated from the pellet of the plasma from an HCV/G-infected tamarin. Viral cDNA was synthesized using a SuperScript reverse transcriptase III (Invitrogen) with random hexamer primers (Invitrogen). After RNase H (Takara) treatment at 37°C, the cDNA mixture was amplified by PCR with LATaq DNA polymerase (Takara) and

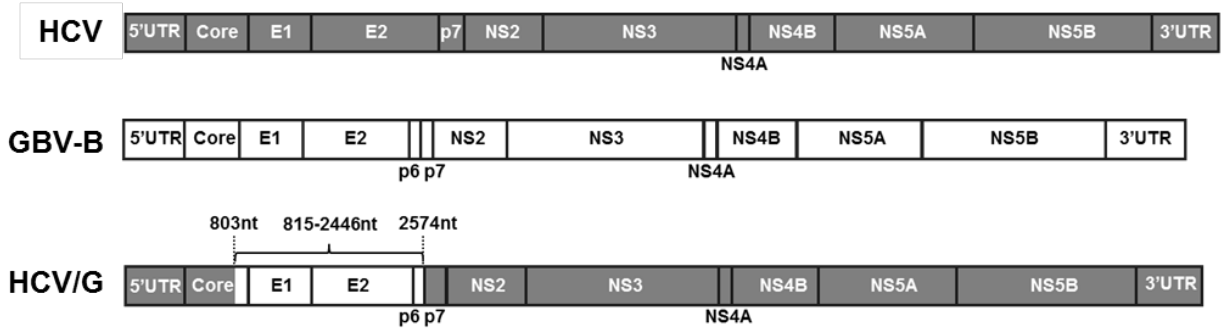
GBS4 [5'- ATGACCCTGTGAATTCCACTCTCCTAC -3'] and HC2790R [5'- ATGCAGCCATCTCCCGGTCCAA -3'] primers for 30 cycles of denaturation at 94°C for 20 sec and extension at 68°C for one minute. A second-round PCR was performed with the GBS4 and HC2769R [5'- CAAGGCGTATGCTCGTGGTGGTAAT -3'] primers for 20 cycles under the same conditions as the first-round PCR. Each PCR primer pair amplified between E2 and NS2 for the first-round and between E2 and p7 for the second-round PCR, respectively. The PCR product was purified using the gel using a QIA-quick gel extraction kit (QIAGEN) and were then cloned into pGEM-T Easy vector (Promega). The PCR products were sequenced with a CEQ-2000XL DNA analysis system (BECKMAN COULTER) with a DTCS quick start kit and HCV/G-specific primers according to the manufacturer's instructions. Sequence data were analyzed with a Sequencher (Gene Codes Corporation) and MacVector (MacVector, Inc.) software packages.

2.4. Results

Construction of a chimeric viral genome using HCV TPF-1 and GBV-B molecular clones

My final goal is to establish a novel, immune-competent, surrogate model using non-human primates for the evaluation of the vaccine candidates targeting NS proteins. I hypothesized that the narrow host range of HCV could be mainly determined at the fusion and/or entry steps of the viral life cycle. If it is the case, substitution of HCV E1 and E2 in the corresponding regions of GBV-B may overcome this hurdle. I therefore sought to construct an HCV/GBV-B chimeric clone (HCV/G) carrying core and NS of HCV and E1 and E2 regions of GBV-B. An HCV/G clone was constructed by swapping C-terminus of core region corresponding a signal peptide sequence, E1 and E2 (804-2573 nucleotides from the 5'UTR) of HCV genotype 1b TPF1 clone for their counterpart elements of GBV-B core signal peptide sequence, E1, E2 and p6 (815-2446 nucleotides) (Fig. 7). According to previous studies, signal peptide and signal peptide peptidase cleavage sites of GBV-B core protein are necessary for productive infection in NWMs; I therefore constructed a chimera containing GBV-B signal peptide sequence in the core region (159). Of note, chimeric GBV-B/HCV containing GBV-B p6 region and HCV p7 region has been shown to replicate efficiently in marmosets following intra-hepatic RNA injection (152). HCV p7 reportedly forms an ion channel and is critical for viral infectivity (160, 161). In addition, the junction between E2 and p6 of GBV-B is a major cleavage site for host signal peptidase, while p6 region is dispensable for the viral replication (162). Considering these data, I constructed a chimera containing GBV-B p6 and HCV p7.

a



b

150aa (789nt) signal peptide core ← E1

ALAHGVRLLEDGVNWATGWFGVHLFVVCLLSLACPCSGARVTDPDNTTILTNCQQR

NQVIYCSPSTCLHEPGCVICADECWVPANPYISHPSNWTGTDSFLADHIDFVMGALVT

CDALDIGELCGACVLVGDWLVRHWLIHIDLNETGTCYLEVPTGIDPGFLGFIGWMAGK

VEAVIFLTKLASQVPYAIATMFSSVHYLAVGALIYYASRGKWYQLLLALMLYIEATSGNPI

RVPTGCSIAEFCSPLMIPCPCHSYLSENVSEVICYSPKWTRPITLEYNNSISWYPYTIPG

ARGCMVKFKNNTWGCCRIRNVPSYCTMGTDVWVNDTRNTYEACGVTPWLTTAWHN

GSALKLAILQYPGSKEMFKPHNWMSGHLYFEGSDTPIVYFYDPVNSTLLPPERWARLP

GTPPVVRGSWLQVPQGFYSDVKDLATGLITKDKAWKNYQVLYSATGALSLTGVTTKA

VVLILLGLCGSKYLILAYLCYLSLCFGRASGYPLRPVLPSQSYLQAGWDVLSKAQVAPF

ALIFFICCYLRCRLRYAALLGFVPM**EA**LE 703aa (2588nt)

E2 ← p6 p6 ←

Fig. 7 Schematic diagram of HCV/G chimeric viral genome

(a) Construction of HCV/G by replacing HCV (black) and GBV-B (white) genomes. The HCV/G chimeric clone was constructed by swapping HCV TPF1 core, E1 and E2 (804-2573 nucleotides from the 5' UTR) counterpart elements with GBV-B core, E1, E2 and p6 (815-2446 nucleotides) containing the core signal peptide. (b) Amino acid sequences at the insertion site of GBV-B core, E1, E2 and p6. The amino acid residues of HCV/G between 150 and 703 are shown. HCV and GBV-B amino acid sequences are indicated in bold and fine, respectively. The signal peptide sequence derived from GBV-B is underlined.

Replication efficiency of HCV/G chimera in Huh7 cells and primary marmoset hepatocytes

I examined the replication efficiency of the HCV/G chimera and compared it with that of HCV TPF1 and its defective mutant clone, TPF1-dGDD, in Huh7 cells (Fig. 8). HCV/G and TPF1, but not TPF1-dGDD, RNA-transfected Huh7 cells efficiently expressed core protein (Fig. 8a). HCV/G exhibited less core protein in the cell culture supernatants than did TPF1, whereas core was undetectable in TPF1-dGDD (Fig. 8b). I then examined the replication efficiency of the viral clones in primary marmoset hepatocytes. HCV/G produced comparable amounts of core protein in the culture supernatants as did TPF1 (Fig. 9). These results suggest that HCV/G is capable of substantially replicating and producing viral particles in both Huh7 cells and marmoset primary hepatocytes.

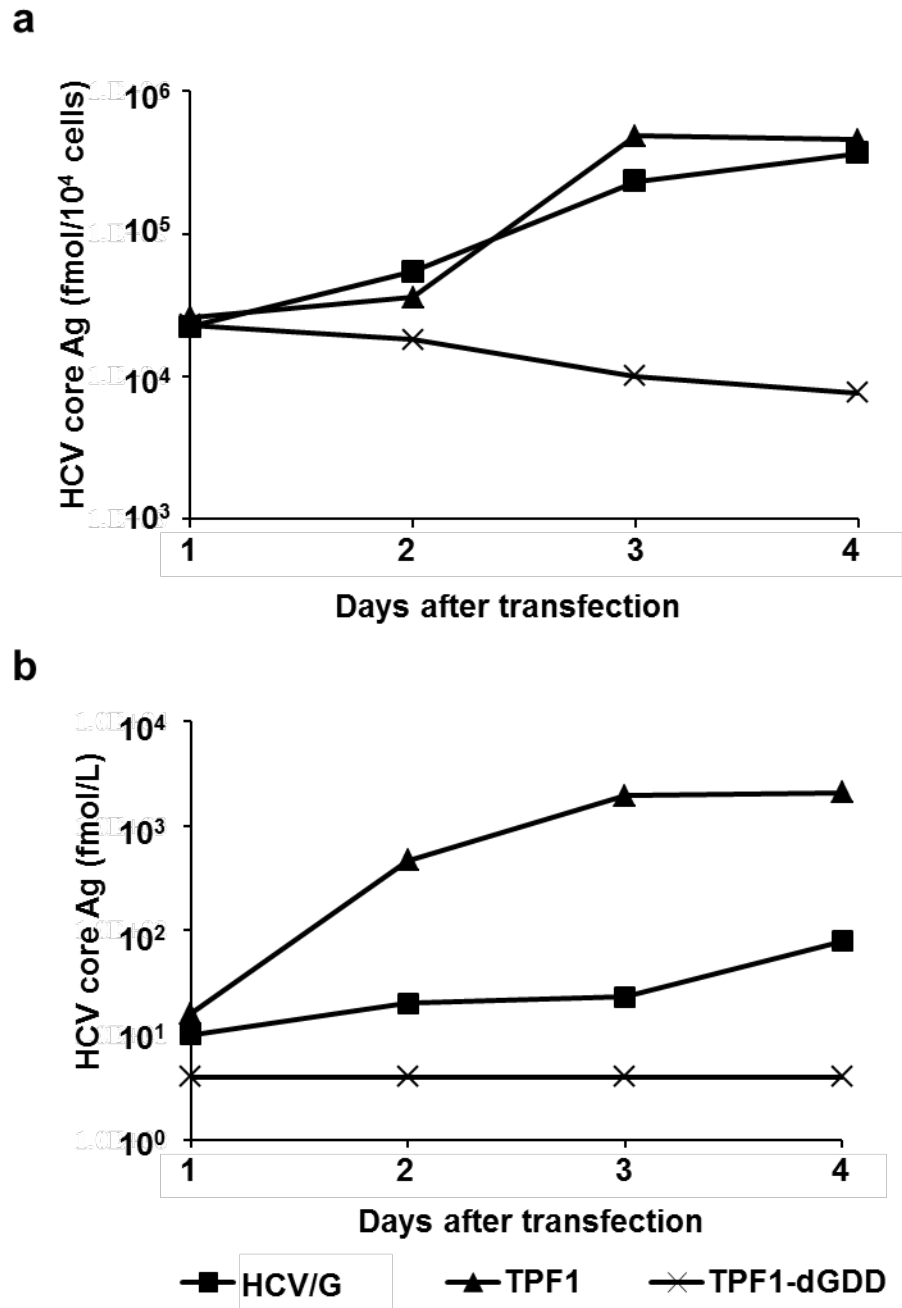


Fig. 8 *In vitro* replication of HCV/G chimera RNA in Huh7 cells

Huh7 cells (5×10^4) were transfected with 3 μ g RNA of HCV/G chimera, wild type HCV (TPF1) and its defective mutant (TPF1-dGDD). The transfected cells (a) and supernatants (b) were evaluated for HCV core antigen amounts by EIA.

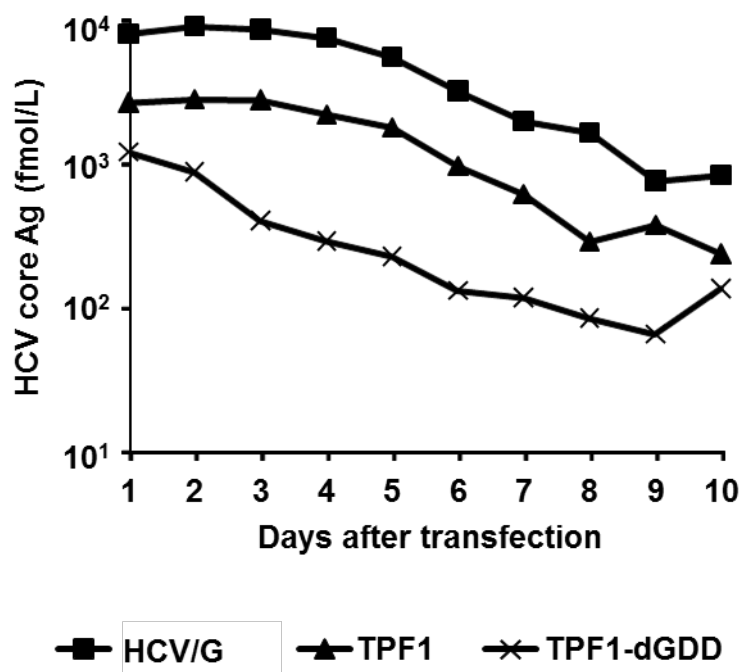


Fig. 9 *In vitro* replication of HCV/G chimera RNA in marmoset hepatocytes

Primary marmoset hepatocytes (1×10^5) were transfected with 3 μ g RNA of HCV/G chimera, wild type HCV (TPF1) and its defective mutant (TPF1-dGDD). The supernatants were evaluated for HCV core antigen amounts by EIA.

Sucrose density gradient analysis of HCV/G chimera

To further characterize the chimera viral particles, I assessed concentrated culture supernatants for the density distribution of the viral core protein and genome RNA. I observed the peak of HCV/G RNA between 1.11 and 1.16 g/ml, whereas the peak of core protein was 1.15 and 1.18 g/ml in NP-40 untreated samples (Fig. 10a). A previous study found the peaks of HCV RNA and core protein in the HCV particles purified from cell culture were approximately 1.15 g/ml (115), which is comparable with the density value of HCV/G chimeric RNA. On the other hand, in NP-40 treated samples, we observed the peaks of HCV/G RNA and core protein between 1.19 and 1.21 g/ml (Fig. 10b). As described previously, detergent treatment removes a lipid envelope from the HCV particles, resulting in the modification of crude populations to the unenveloped viral particles and shift of both viral RNA and core protein to higher density (163, 164). These results suggested that the NP-40 treated viral particles were associated with HCV/G RNA. In a study of HCV particles collected from a patient and treated with NP-40, the peak of HCV RNA was reportedly 1.18 g/ml (165), which is consistent with my result.

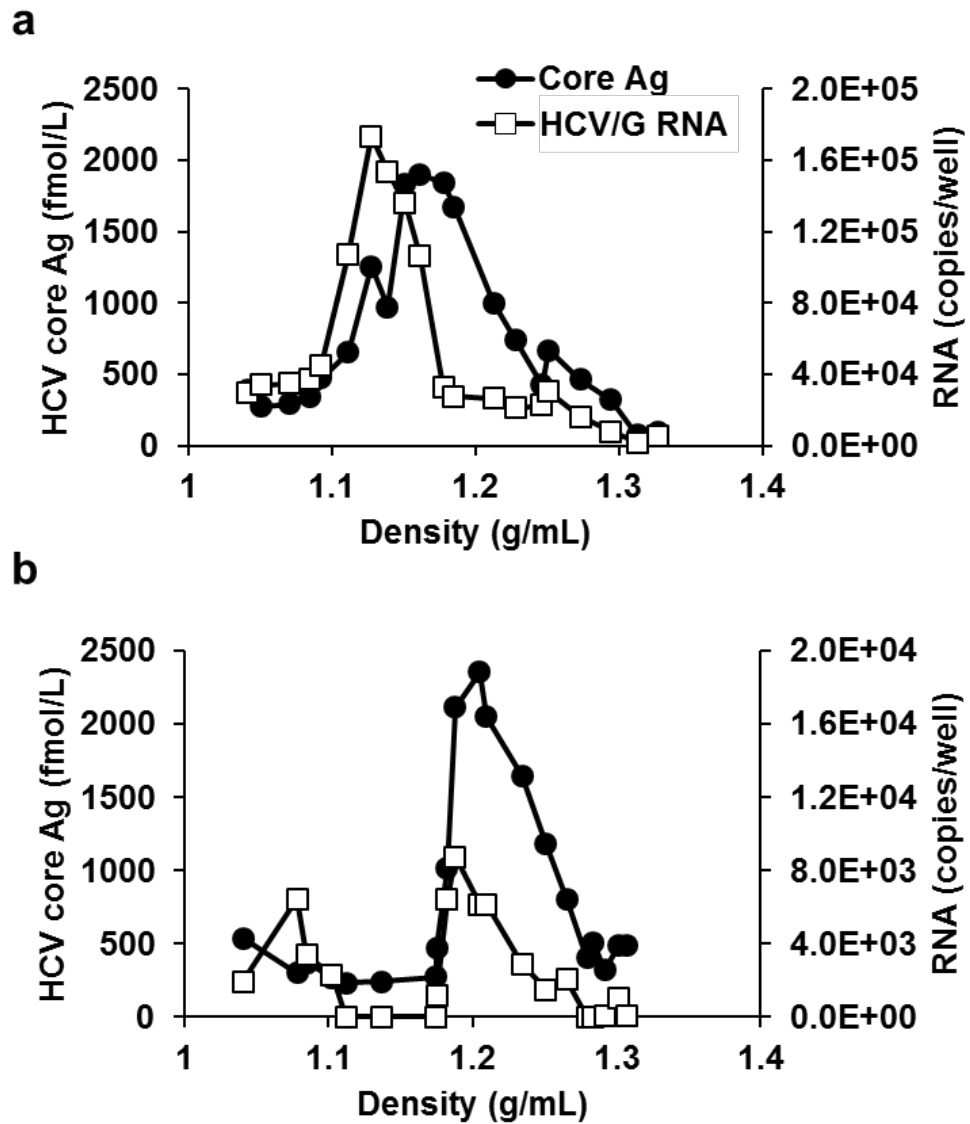


Fig. 10 Density distribution of HCV/G core protein and the viral genomic RNA from culture supernatants treated with or without NP-40 by sucrose gradient centrifugation

NP-40 untreated (a) and treated (b) concentrated supernatants collected from HCV/G RNA-transfected primary marmoset hepatocytes were subjected to ultracentrifugation on a stepwise sucrose gradient. After centrifugation, 20 fractions were collected. The supernatants were evaluated for HCV core antigen amounts by EIA and for HCV/G RNA by real-time PCR.

Inoculation of HCV/G chimeric RNA in a tamarin

I then asked whether the HCV/G chimera could replicate *in vivo*. As an experimental control, I inoculated serum containing GBV-B (3.3×10^7 copies) was intravenously inoculated into a tamarin. I detected GBV-B RNA for 12 weeks in both plasma and PBMC, followed by acute clearance (Fig. 11a and b). I observed increased plasma ALT levels throughout the period of viremia (Fig. 11c). In the case of a monkey inoculated with HCV/G genomic RNA, viral RNA was detectable intermittently throughout an observation period for more than two years, while the viral load was lower than that of GBV-B (Fig. 11d and e). I detected no significant increase in ALT levels except for 118 and 126 weeks after inoculation (Fig. 11f).

Finally, I sought to ascertain if plasma viral RNA was derived from the chimeric virus particles. I ultracentrifuged a plasma sample at 73 weeks after inoculation and used the pellet fraction for viral RNA extraction. I amplified the chimera-specific sequence from the pellet by nested RT-PCR and confirmed the cDNA sequence of the amplicon to be of HCV/G genome (data not shown). These results indicated that HCV/G chimera persistently replicates and produces viral particles *in vivo*.

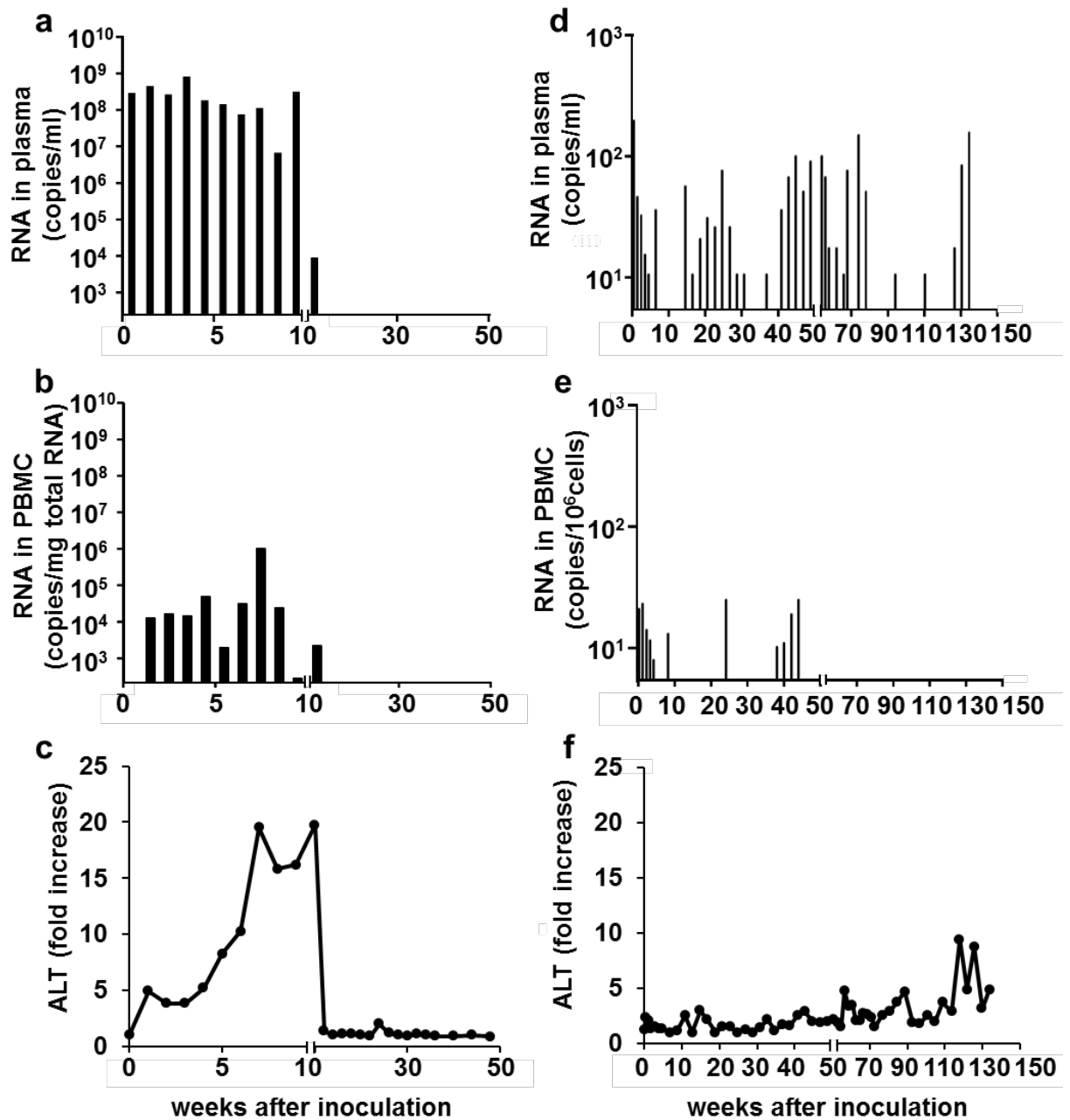


Fig. 11 Kinetics of virus loads and ALT levels in tamarins inoculated with GBV-B or HCV/G chimera

GBV-B infectious serum was intravenously inoculated into a tamarin, viral RNA copies in plasma (a) and PBMC (b) and plasma ALT levels (c) in the monkey were measured until 50 weeks after inoculation. The cutoff value for viral RNA quantification was 250 copies/ml. HCV/G RNA was inoculated into a tamarin intrahepatically. Viral RNA copies in plasma (d) and PBMC (e) and plasma ALT levels (f) in the monkey are shown until 134 weeks after inoculation. The cutoff value for viral RNA quantification was 5 copies/ml. ALT levels are indicated as fold increase in the mean ALT levels measured periodically before inoculation.

2.5. Discussion

My final goal is to establish a novel immune-competent surrogate model for evaluating the prophylactic and therapeutic efficacy of CTL-inducible vaccines targeting NS proteins. I thus sought to construct an HCV/GBV-B chimera that is replication-competent in NWMs. Based on our intermittent detection of chimeric viral RNA in plasma and PBMC for more than two years and detection of HCV/G genome from pellet fraction of plasma at 73 weeks after inoculation, I concluded that the chimera was able to replicate persistently *in vivo*. My results will help establish a novel non-human primate model for HCV infection on the basis of the HCV/G chimera in the major framework of HCV genome. This model will be able to evaluate hepatitis C vaccines which induce cellular immune responses against HCV core or nonstructural proteins.

It remains to be elucidated whether HCV/G chimera is capable of producing infectious virus and establish multiple rounds of viral infection *in vivo*. It will be important to further evaluate reproducibility of the results obtained in this study and to examine if the *in vivo* passage could improve the competency of the viral replication and infectivity. HCV/G genome-transduced hepatocytes may possibly produce noninfectious viral particles for years. However, it is unlikely because the average life span of hepatocytes is several months (166), although it is possible that, viral production is attributed to liver stem cells (167). Our detection of chimeric RNA in PBMC (Fig. 11e) suggests that chimeric virus produced in the liver and may be re-infected with lymphocytes as described previously for HCV and GBV-B (70, 168, 169). Even if the chimera is capable of producing infectious virus, the viral infectivity is likely remarkably low. This inefficient infectivity may be caused by either failure of core

and/or NS proteins derived from HCV to function appropriately or to GBV-B envelope protein and/or tamarin apolipoproteins not being fully packaged into viral particles because of insufficient association with HCV core and/or NS proteins (170, 171). Because monkey hepatocyte-derived cell lines permissive for GBV-B and the chimeric virus are so far unavailable except for primary hepatocytes, which are impractical, establishment of such monkey hepatocyte-derived cell lines would help improve infectivity of the chimeric virus.

Although I detected HCV/G chimeric RNA intermittently, I was unable to detect antibodies against HCV/G (data not shown). This may be because antigenic stimulation was not strong enough to induce antibodies. In fact, the previous findings reported in my laboratory, which in the course of persistent GBV-B infection, anti-core antibodies were not induced when the plasma viral loads were lower than 10^4 viral copies/ml is consistent with the present result (66). The fact that the viral loads were low and antibodies against HCV/G were undetectable is comparable to occult HCV infection (OCI) since OCI is reported to be defined by the presence of HCV RNA in hepatocytes without detectable HCV RNA in serum; antibody against HCV is positive or negative (172-175). My animal model may contribute to the understanding of the immunological and virological mechanisms by which OCI is maintained.

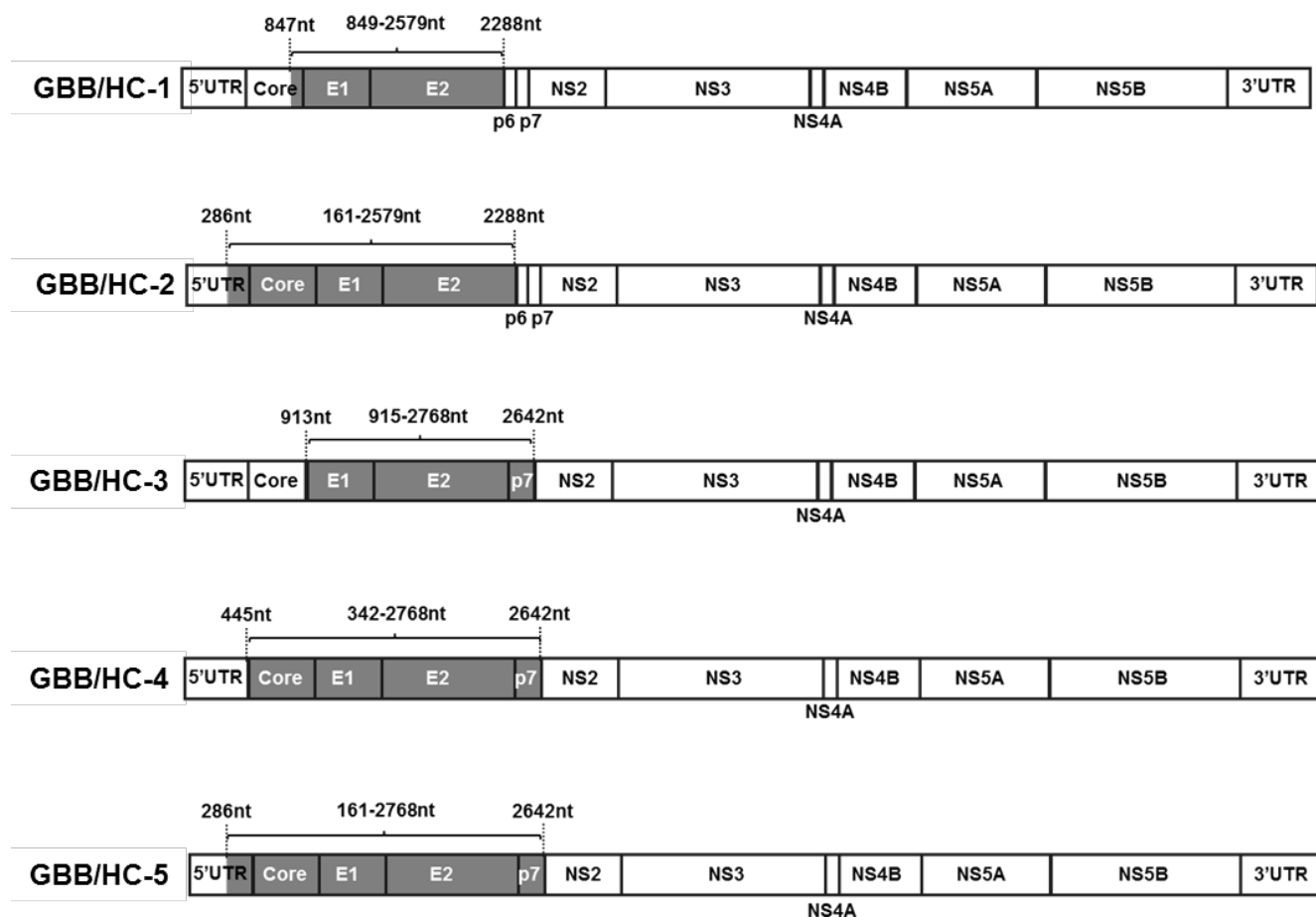
My results in this study indicate that HCV/G chimeric virus carrying GBV-B envelope gene persistently replicates in a tamarin, suggesting that E1 and E2 derived from GBV-B are critical for persistent replication in NWMs. However, it is possible that the HCV clone TPF1 itself is able to replicate in NWMs. The compatibility of envelope proteins of HCV and GBV-B in human and monkey cells needs to be further elucidated. During I analyzed these data, Li et al. reported that a chimeric virus composed of entire

envelope E1, E2 and p7 regions with or without the core region of HCV genes exhibited efficient replication capacity and developed chronic infection in NWMs (176). It strongly suggests that HCV E1 and E2 may not determine the non-permissiveness of NWMs to HCV infection. The narrow host range of HCV could not be determined only at the fusion and/or entry steps of the viral life cycle. The chimeras composed of the entire envelope E1, E2 may be useful for evaluating vaccine candidates targeting E1 and E2 proteins.

For further investigating HCV/GBV-B chimeric construct, I established new chimeric constructs using HCV TPF-1 and GBV-B (GBB/HC-1, 2 in Supplementary Fig. 4). These chimera were constructed to investigate whether (i) HCV envelope and/or core protein can alter GBV-B counterparts, (ii) signal peptide sequences and signal peptide peptidase should be consistent with virus of E1 or not. A previous study described that HCV internal ribosome entry site (IRES) domain III in 5'UTR was able to be an alternative to GBV-B counterpart to infect tamarin (150). The animal model infected by a chimera which codes HCV envelope protein has an ability to evaluate NAb reaction induced by hepatitis C vaccine candidates. GBB/HC-1, 2 chimeras containing HCV genes coding for structural proteins, core-E2 or 5'UTR-E2 substituted for the counterparts of GBV-B were constructed and transcribed by T7 RNA polymerase with a MEGAscript T7 kit (Ambion). The two kinds of synthesized RNA (each 110 µg) were mixed and inoculated into marmosets intrahepatically. The replication was detected in marmosets and the replicated virus predominantly was GBV-B/HC-2 which is containing HCV 5'UTR domain III-E2. These results suggest that HCV domain III of IRES can be alternative and effective even in marmosets. GBV-B/HC-1, 2 caused acute infection but did not develop chronic infection. So now I am preparing new three

chimeric constructs to refine chimera constructs coding HCV structural protein to obtain an animal model which has ability to evaluate NAb reaction induced by hepatitis C vaccine candidates (GBB/HC-3, 4, 5 in Supplementary Fig. 4). The first is constructed by swapping GBV-B E1-p7 for HCV E1-p7, while the second is constructed by swapping GBV-B core-p7 for HCV core-p7. The third is constructed by swapping GBV-B 5'UTR-p7 for HCV 5'UTR-p7. I will investigate the infectivity of these chimeras to refine chimera construct for higher and longer viremia and clinical symptom in NWMs.

I believe that in near future these analyses will make it possible to construct monkey-tropic HCV with only small modifications including a specific gene or a few amino acids derived from GBV-B, which is efficiently replication-competent *in vivo*. This monkey-tropic HCV should be most suitable animal model for evaluating prophylactic and therapeutic effects of hepatitis C vaccines.



Supplementary Fig. 4 Schematic diagram of new chimeric viral genome

Constructions of GBB/HC-1, 2, 3, 4, 5 by replacing HCV (black) and GBV-B (white) genomes. The GBB/HC chimeric clones were constructed by swapping GBV-B 5'UTR, core, E1, E2, p6 and p7 counterpart elements for HCV TPF-1 5'UTR, core, E1, E2 and p7.

Chapter 3

Comparative analyses of humoral and cellular immune responses in acute and chronic GB virus B infection

3.1. Abstract

It has been shown that humoral and cellular immunity has critical roles in the control of hepatitis C virus (HCV) replication and also the development of chronic hepatitis. However, the precise kinetics in the induction of antiviral antibodies and cytotoxic cells as well as their implication in the virus dynamics through the entire HCV infection period including its acute stage are not fully understood. One of the reasons is the difficulty to obtain periodical samples of HCV-exposed individuals from acute stage of infection without receiving anti-viral treatments. In this point of view, the animal model of experimental HCV infection would be the key to solve this problem. Chimpanzees have been the only animal model that permits HCV infection and induce antiviral immune responses, while the use of the animal for invasive research has recently been prohibited. GB virus B (GBV-B) is closely related to HCV and experimental GBV-B infections of marmosets and tamarins have been shown to develop acute and chronic hepatitis which is comparable to that caused by HCV infection. It is expected that the GBV-B-infected model may bring important insight to clarify the detailed mechanisms of persistent infection and disease development. Furthermore, the insights may be applied to improve the animal model for HCV study.

In this study, I analyzed that the differences of humoral and cellular immune responses in acute and chronic phases of GBV-B infection. Anti-E2, core and NS3 antibodies were induced in the GBV-B-infected monkeys with acute clearance, which crossed over the decrease of plasma viral RNA and alanine aminotransferase (ALT) levels. Unexpectedly, the antibody responses in the persistently infected monkeys were much delayed in some cases irrespective of the levels of plasma viral RNA which were

theoretically sufficient to induce efficient antibody responses. Furthermore, subsets of Natural Killer (NK) cells expressing cytotoxic markers ($CD3^- CD16^+ CD56^{dim} CD335^+ CD159a^+$) increased in the cell number in the course of the acute clearance. On the other hand, the rate of the cytotoxic NK cells in the chronically infected monkey remained low as much as that in naïve and the GBV-B-cured monkeys. These results suggest that the induction of antiviral responses including the antibodies and cytotoxic NK cells may be associated with the acute clearance and that the inefficient induction may involve in the persistent GBV-B infection.

3.2. Introduction

In 2nd chapter, I demonstrated that the chimeric virus (HCV/G) composed of hepatitis C virus (HCV) and GB virus B (GBV-B) caused persistent replication in a red-handed tamarin (*Saguinus midas*) *in vivo* for more than two years. However, it was still needed to improve replication efficiency and virulence of the chimera in order to apply this animal model to the evaluation of hepatitis C vaccine candidates. It has been already reported that GBV-B causes acute or chronic infection (67, 69, 154, 177) and that some chronically infected tamarins or marmosets caused hepatitis C-like disease (66, 68). So far, immunological mechanism(s) by which the viral replication is efficiently regulated in the case of acute clearance remains to be elucidated. Previous reports showed that rapid induction of neutralizing antibodies (NAb) may contribute to early control of HCV replication, while contradict findings were also reported. The early NAb may not be sufficient for a long-term protective immunity in chimpanzees (143) and humans (141, 142), even in the individuals exhibiting cross-reactive NAb in the chronic phase. The precise kinetics in the induction of antiviral antibodies and its implication in the virus dynamics through the entire course of HCV infection including its acute stage are not fully understood. One of the reasons for this discrepancy is the difficulty to perform longitudinal study by using periodical samples of HCV-exposed individuals from the acute stage of infection without receiving anti-viral treatments. In this point of view, the animal model of experimental HCV infection would be the key to solve this problem.

Summarizing to the previous data accumulated in my laboratory, approximately 10% of GBV-B-infected monkeys caused chronic infection (Higashino et al., in preparation). In this study, I analyzed both humoral and cellular immune responses in

New World monkeys (NWMs) in the acute and chronic GBV-B infection. Analyses of antibody responses against E2, core and NS3 in GBV-B infected monkeys, the antibody titers were lower and the induction was much delayed in chronically infected monkeys as compared with those of the sub-acute infection.

As well as NAb, some previous reports showed that Natural Killer (NK) cells, the major effector cells of the innate immune system, play a role in the early control of HCV infection, although the role of NK cells is still controversial. Approximately 30% of intrahepatic lymphocytes are NK cells and the percentage appears to increase to more than 50% in the patients with liver disease (178, 179). NK cells have been divided into two functional subsets; one is CD3⁻ CD56^{high} CD16⁻ NK cells which secrete cytokines such as IFN γ but display poor natural cytotoxic activity, while the other is CD3⁻ CD56^{dim} CD16⁺ NK cells which have high cytotoxic capability such as secretion of perforin but poor production of cytokines (180). In the previous studies, relationship between NK cells and HCV outcome, especially correlation between CD335 (alias, NKp46)/NKp30, members of natural cytotoxicity receptor family, and HCV outcome is controversial. Some reports indicated that low expressions of CD335/NKp30 on NK cells in the acute phase are associated with HCV clearance, whereas, others reported that expressions of CD335/NKp30 on NK cells in chronic phase were lower than healthy control and that expressions of CD335/NKp30 in patients who cleared HCV or who were uninfected with high HCV exposure were normal and high, respectively (181-184). It is also reported that intrahepatic CD335^{high} NK cells was inversely correlated with HCV RNA levels and fibrosis stage (178). It is practically difficult to perform longitudinal study regarding HCV-exposed while untreated individuals from the acute stage of infection as described above, therefore this question remains to be

unsolved. I thus sought to examine the kinetics of NK subsets in NWMs infected with GBV-B. I thought that comparison between monkeys with acute clearance and chronic hepatitis would help understanding the functional significance of NK cells.

3.3. Materials and Methods

Animals

The plasmas in eight red-handed tamarins or red-bellied tamarins (*Saguinus labiatus*, weighing 385-592 g) and nine common marmosets (*Callithrix jacchus*, weighing 245-450 g) were used in this study. These monkeys were reared at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Ibaraki, Japan or Primate Research Institute, Aichi, Japan. Animal experiments were conducted in accordance with the protocols of experimental procedures that were approved by the Animal Welfare and Animal Care Committees of the Primate Research Institute of Kyoto University and National Institute of Biomedical Innovation, Japan. All animals were caged individually at $27 \pm 2^{\circ}\text{C}$ in $50 \pm 10\%$ humidity with a 12h light-dark cycle (lighting from 7:00 to 19:00). All animals were fed twice a day with a standard tamarin/marmoset diet supplemented with fruit and mealworm. Water was given *ad libitum*. Blood of monkeys was collected under anesthesia (ketamine, <10 mg/head, intramuscularly).

Biochemical Test

The basic biochemical test regarding alanine aminotransferase (ALT) level in plasmas of the tamarins was measured by a DRI-CHEM7000 (FUJIFILM).

Detection and Quantification of Viral RNA

Viral RNA in plasma samples was purified using a QIAamp Viral RNA Mini kit (QIAGEN). Two hundred microliters of plasma was concentrated into 50 μl RNA

extracts. Two microliters of RNA extracts were quantified by real-time PCR using a TaqMan RNA-to-Ct 1-Step Kit (TaqMan). To detect GBV-B core region, the primers GBV-B For [5'- AACGAGCAAAGCGCAAAGTC -3'] and GBV-B Rev [5'- CATCATGGATACCAGCAATTTTGT -3'] were used.

Antibody titers

The monkey plasma samples were evaluated for anti-GBV-B E2, core and NS3 antibodies by ELISA as described previously (70, 185). Briefly, the TrpE-E2 (aa 20 to 224) fusion protein, TrpE-core (aa 1 to 132) fusion protein and TrpE-NS3 (aa 1135 to 1378) fusion protein, representing a portion of NS3 identified as being immunogenic in infected animals (186), was expressed in *E. coli* (187) to serve as an antigen to generate polyclonal rabbit antisera. The plasma samples were evaluated for the antibodies against GBV-B E2, core and NS3 by ELISA.

Flow cytometry

Flow cytometric analysis was performed as previously described with a slight modification (188). For analysis of immune cells in an acute GBV-B-infected marmoset, a chronically GBV-B-infected tamarin and naïve marmosets, PBMC were isolated from whole blood by layering onto Ficoll-Paque PLUS (GE Healthcare Life Sciences) and centrifugation. These samples were hemolyzed by ACK lysis buffer (0.15M NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA) at room temperature for five minutes and washed by phosphate buffered saline (PBS) containing 1% fetal bovine serum (FBS). Isolated PBMC were stained with combination of fluorescence-conjugated antibodies: APC-conjugated anti-CD3 (SP34-2: BD Biosciences), PerCP-Cy5.5-conjugated

anti-CD4 (L200: BD Biosciences), PE-conjugated anti-CD8 (T8-RD1: BECKMAN COULTER), APC-conjugated anti-CD62L (145/15: Miltenyi Biotec), PE-Cy7-conjugated anti-CD95 (DX2: BioLegend), FITC-conjugated anti-CD20 (H299: BECKMAN COULTER), PE-conjugated anti-IgD (polyclonal antibody: Southern Biotech), APC-conjugated anti-CD27 (MT-271: BioLegend), PerCP-Cy5.5-conjugated anti-CD16 (3G8: BioLegend), PE-Cy7-conjugated anti-CD56 (NCAM16.2: BD Biosciences), PE-conjugated anti-CD335 (BAB281: BECKMAN COULTER), APC-conjugated anti-CD159a (Z199: BECKMAN COULTER), PE-conjugated mouse IgG1 (IS5-21F5: Miltenyi Biotec), PerCP-Cy5.5-conjugated mouse IgG1 κ (MOPC21: BioLegend) and APC-conjugated mouse IgG1 κ (MOPC21: BioLegend). After washing with a sample buffer containing PBS and 1% FBS, the labeled cells were resuspended in a fix buffer containing PBS and 1% formaldehyde. Expression of the immunolabeled molecules on the lymphocytes was analyzed with a FACSCanto II flow cytometer (Becton Dickinson). The data analysis was conducted using FlowJo software (Treestar, Inc.).

Statistical analyses

Statistical analyses of antibody titers were performed using Welch's *t*-test.

3.4. Results

Induction of antibody titers against GBV-B E2, core and NS3 in tamarins and marmosets which were cured in the acute phase of GBV-B infection

To investigate host factors which restrict replication efficiency and prevent viruses from causing chronic infection, I analyzed that the induction of antibodies in acute/chronic GBV-B infection. Previously, four red-handed tamarins, two red-bellied tamarins and seven common marmosets were inoculated with GBV-B ($1 \times 10^{3-9}$ /head or GBV-B RNA) intravenously or intrahepatically. The kinetics of GBV-B RNA and ALT levels in these animals were shown in Fig. 12. In the animals with acute clearance of the infection, ALT levels were increased in parallel with the viral RNA levels especially in Tm03-007 and Tm03-008 (Fig. 12a and b). I used the plasma samples from the monkeys for evaluation of antibody titers. Anti-E2, anti-core and anti-NS3 antibodies were induced at 10-20 weeks after virus inoculation, which crossed over the decrease of plasma viral RNA. The antibody titers reached to the peaks within 20 weeks after inoculation followed by gradual reduction when the viral RNA decreased and undetectable.

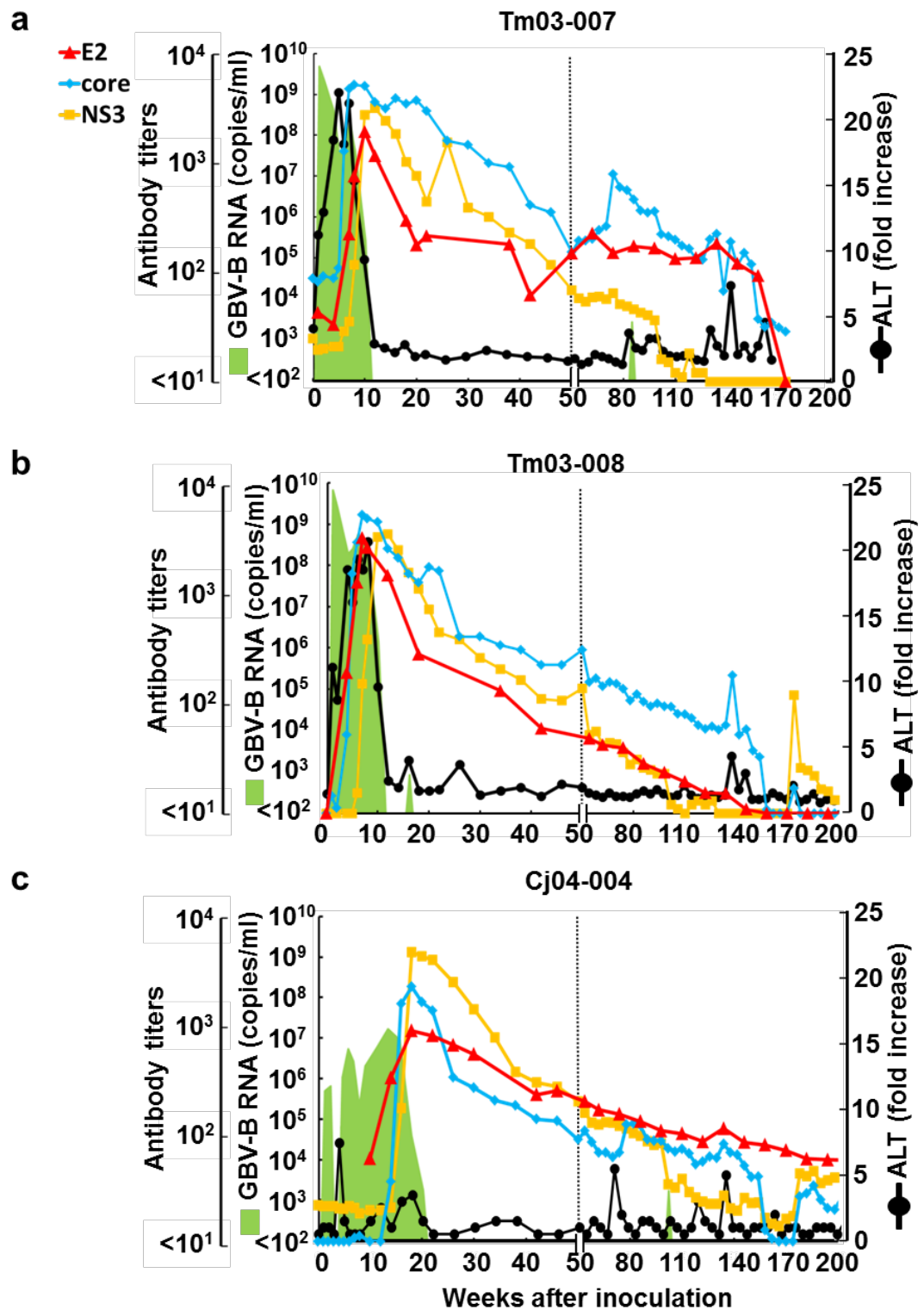


Fig. 12 Kinetics of acute GBV-B infection

GBV-B RNA in plasma is indicated as green. ALT, and antibodies against E2, core, NS3 are indicated as black, red, blue and yellow lines, respectively. Data of tamarins; Tm03-007 (a) and Tm03-008 (b), and marmoset; Cj04-004 (c) are indicated. The dot-lines at 50 weeks after inoculation is indicated the border of different interval of y-axis.

Delay in the induction of antibody titers against GBV-B E2, core, NS3 in the monkeys with GBV-B chronic infection

I employed two red-handed tamarins and two common marmosets with chronic GBV-B infection. Interestingly, two monkeys, Tm06-018 and Cj05-004, developed progressive hepatitis, while the others, Tm05-011 and Cj05-002, resulted in spontaneous clearance until 200 weeks after inoculation, with the ALT levels increased intermittently during viremia (Fig. 13). In contrast to the monkeys with acute clearance, the antibody responses in these with chronic infection were much delayed and it took up to 1-3 years to reach plateau of the antibody titers. The kinetics of antibody responses differed among chronically infected individuals. Especially, the induction of anti-E2 antibodies were delayed in the monkeys developed progressive hepatitis as compared with those resulted in spontaneous clearance.

Fig. 14 indicated that the weeks when these antibody titers reached plateau in those with acute clearance and chronic infection. No obvious differences were observed among the monkeys with acute clearance in the periods as the titers reached the peaks. The induction of anti-E2, core and NS3 antibodies were delayed in the chronic infection cases as compared with those with acute clearance.

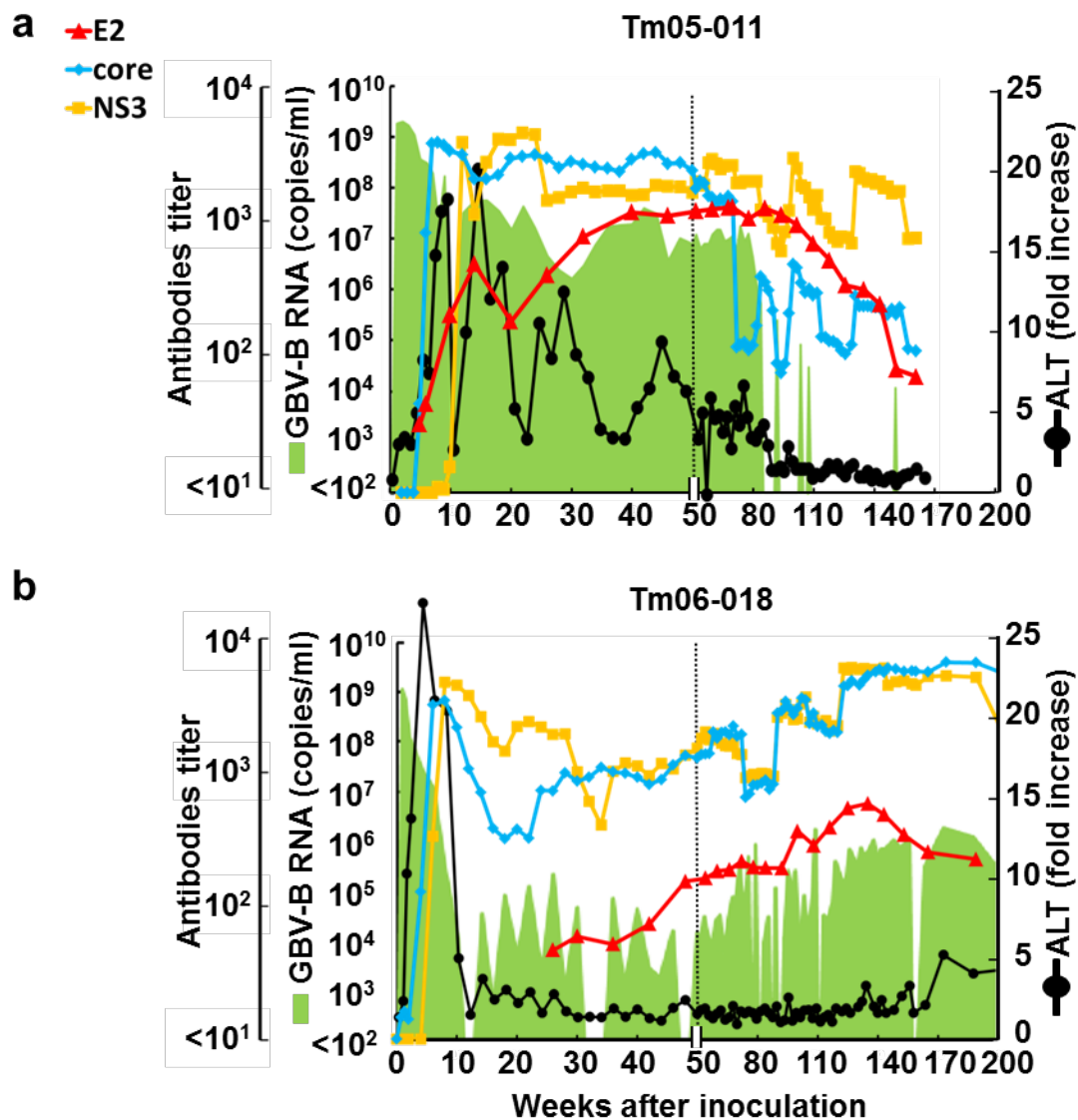


Fig. 13 Kinetics of chronic GBV-B infection

GBV-B RNA in plasma is indicated as green. ALT, and antibodies against E2, core, NS3 are indicated as black, red, blue and yellow lines, respectively. Data of tamarins; Tm05-011 (a) and Tm06-018 (b), and marmosets; Cj05-002 (c) and Cj05-004 (d) are indicated. The dot-lines at 50 weeks after inoculation is indicated the border of different interval of y-axis.

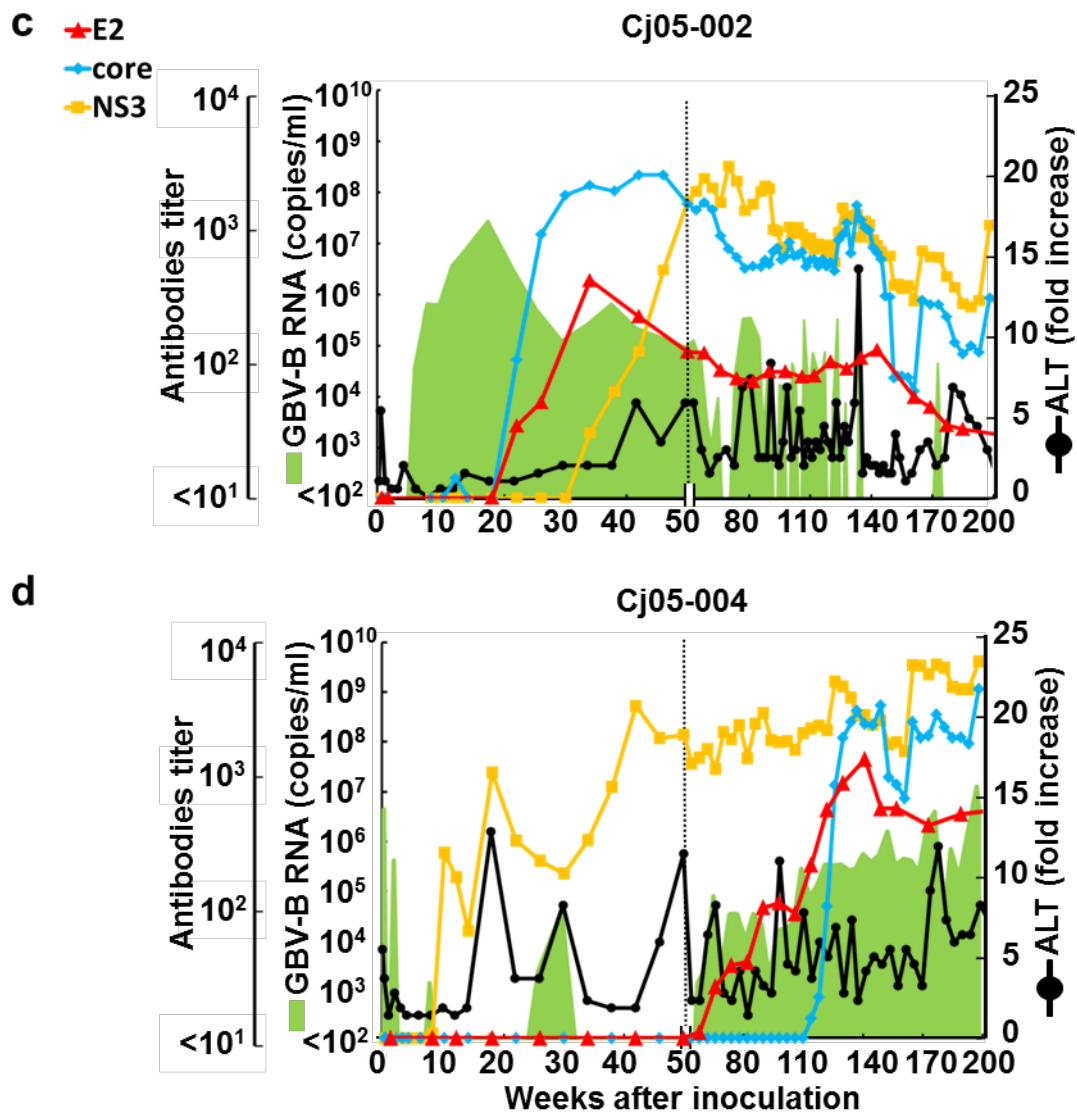


Fig. 13 (continue)

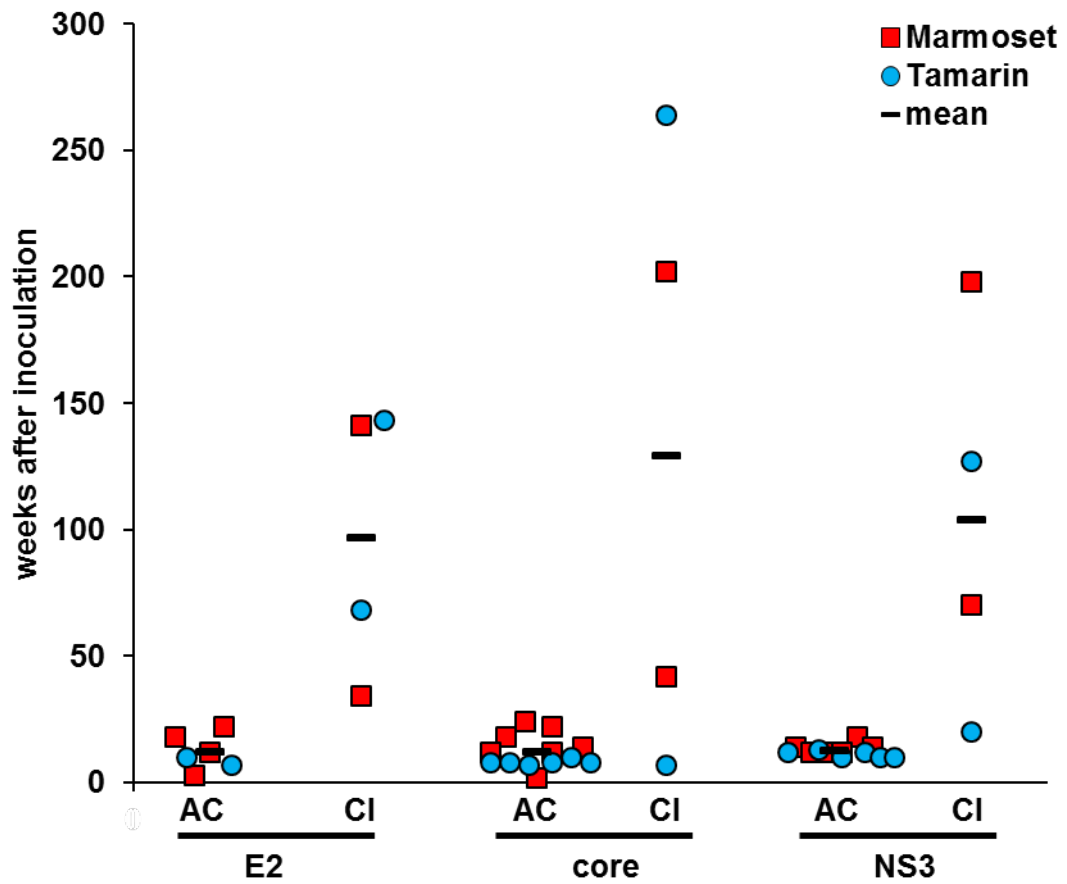


Fig. 14 Weeks when antibody titers reached plateau in the monkeys with acute clearance (AC) and chronic infection (CI)

Anti-E2, core and NS3 antibodies in marmosets are indicated as red boxes, antibodies in tamarins are indicated as blue circles. Means in the titers of each antibody are indicated as black lines.

Kinetics of lymphocytes in a marmoset with acute GBV-B infection and comparison between acute clearance and chronic infection

As well as humoral immunity like the induction of antibodies, cellular immunity also possible to be related to restricting replication efficiency and preventing viruses from causing chronic infection. In addition, the reports are rare in which it is investigated that the kinetics of lymphocytes in early phase of HCV infection. The kinetics in GBV-B infection is also not yet fully understood. Thus, I investigated the kinetics of lymphocytes in a marmoset with acute GBV-B infection and compared with chronic infection.

A marmoset was inoculated GBV-B (1×10^4 /head) intravenously and then, GBV-B RNA, ALT level and antibody titers against E2, core and NS3 were evaluated (Fig. 15a). Lymphocytes ($CD4^+$ T cells, $CD8^+$ T cells, B cells and NK cells) were monitored by flow cytometry (Fig. 15b). Monitored T cells were naïve T cells ($CD62L^+ CD95^-$), effector T cells ($CD62L^- CD95^+$) and central memory T cells ($CD62L^+ CD95^+$) in $CD3^+ CD4^+$, $CD3^+ CD8^+$ T cells and $CD3^+ CD4^+ CD8^+$ T cells. Monitored B cells were naïve B cells ($IgD^+ CD27^-$), IgM memory B cells ($IgD^+ CD27^+$) and class switch memory B cells ($IgD^- CD27^+$) in B cells ($CD3^- CD20^+$). It is known that class switch memory B cells produce IgG, IgM and IgA, whereas IgM memory B cells predominantly produce IgM (189). Monitored NK cells were $CD3^- CD56^{dim} CD16^+$ NK (cytotoxic T lymphocytes NK; CTL-NK) cells subset which has cytotoxic activity, $CD3^- CD56^{high} CD16^-$ NK cells subset which has cytokine-producing activity. The both NK cells express CD335 and/or CD159a. CD335 is a member of natural cytotoxicity receptor family, while CD159a is inhibitory marker and co-expressed with CD335 on NK cells in HCV-infected patients (178).

GBV-B RNA showed less than 1×10^8 copies/ml and the virus was excluded 12 weeks after inoculation. The kinetics of lymphocytes (Fig. 15b) did not change dramatically through this experiment. In the previous report about dengue-infected marmosets, CD4/CD8 central memory T cells were greatly induced, and naïve T cells were reduced in the acute phase (92), while no significant changes of these subsets were observed in this study (data not shown). In cirrhotic patients related to HCV infection, CD27⁺ B cells showed less frequent than healthy donors (190), while no difference in this acute GBV-B infected marmoset (data not shown). Interestingly, CD335⁺ cells subsets, CD159a⁺ cells subsets and CD335⁺ CD159a⁺ cells subsets in CTL-NK (CD3⁻ CD56^{dim} CD16⁺ cells) were increased after virus load increased, and then paralleled with virus load, although total NK cells did not changed significantly (Fig. 15c left).

In comparison with the NK subsets in acute clearance, the subsets in a tamarin with chronic infection (Fig. 15c right) were low as much as that in naïve marmosets (Fig. 15 middle) and the GBV-B-cured marmoset after the acute phase (Fig. 15c left). These subsets in the chronically infected tamarin were analyzed 333 weeks after GBV-B inoculation. The GBV-B RNA was 3×10^6 copies/ml at the time. These results suggest that cytotoxicity in NK cells is critical to exclude GBV-B in the acute phase.

Taking these findings into consideration, it is implied that the delay of time and weakness of the antibody inductions and low cytotoxicity in NK cells are related to inducing chronic GBV-B infection.

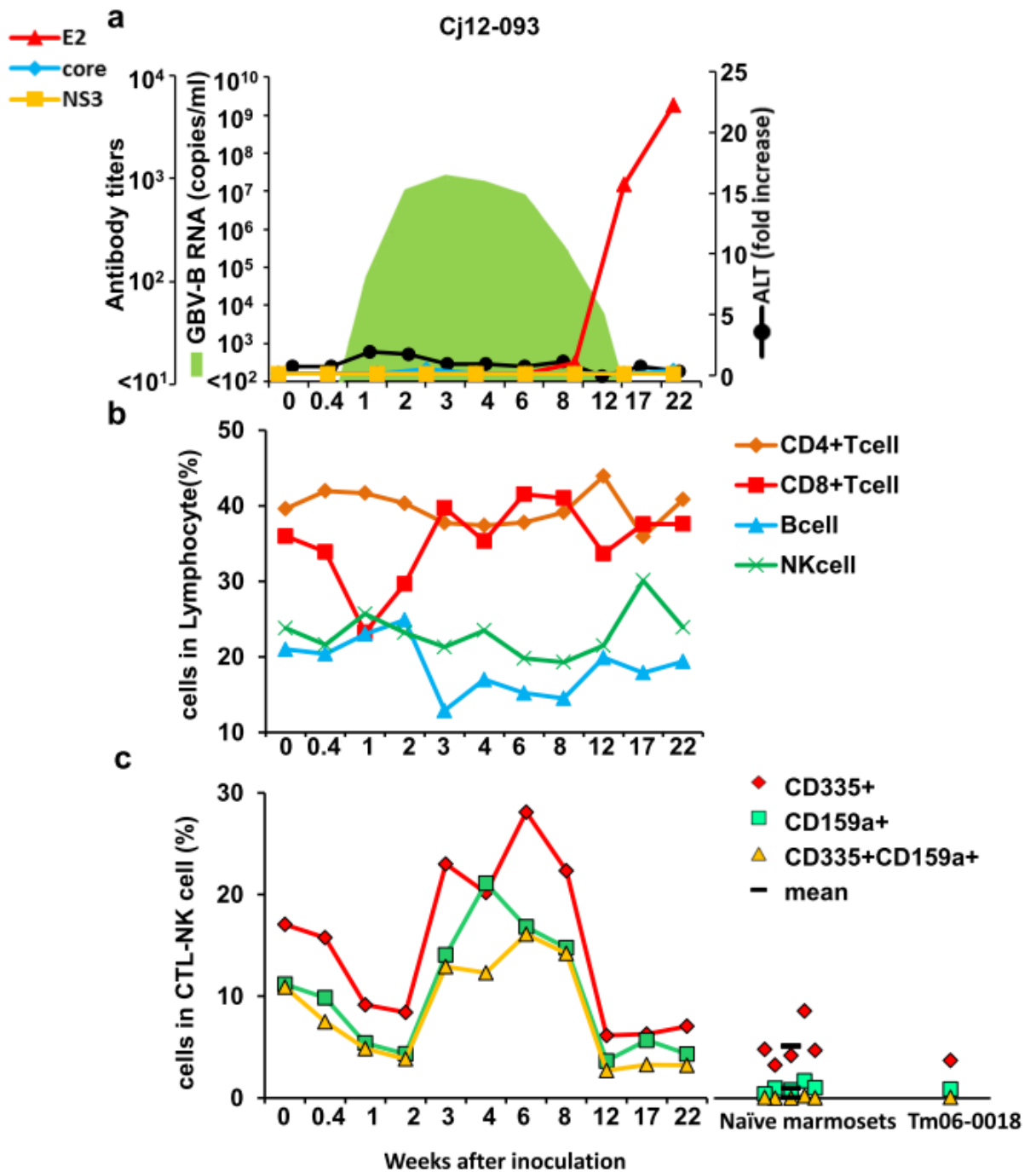


Fig. 15 kinetics of acute GBV-B infection and lymphocytes in a marmoset

(a) GBV-B RNA in plasma is indicated as green. ALT, and antibodies against E2, core, NS3 are indicated as black, red, blue and yellow lines, respectively. (b) Periodical kinetics for the fluorescence intensity of CD4⁺ T (CD3⁺ CD4⁺), CD8⁺ T (CD3⁺ CD8⁺), B (CD3⁻ CD20⁺) and NK (CD3⁻) cells in lymphocyte subsets are indicated as orange, red, blue and green lines, respectively. (c) Periodical kinetics for the fluorescence intensity of CD335⁺, CD159a⁺ and CD335⁺ CD159a⁺ cells subsets in CTL-NK cells (CD3⁻ CD56^{dim} CD16⁺) are indicated as red, green, and yellow lines, respectively. These cells subsets in chronically infected tamarin (Tm06-018) and naïve marmosets are indicated right and middle. Means of each subset in naïve marmosets are indicated as black lines.

3.5. Discussion

In this study, I analyzed that the differences of humoral and cellular immune responses in acute and chronic phases of GBV-B infection. The induction of anti-E2, core and NS3 antibodies in chronically infected monkeys were much delayed and the titers were low in some infected monkeys. In addition, NK cells expressing cytotoxic markers (CD3⁻ CD16⁺ CD56^{dim} CD335⁺ CD159a⁺) increased after viremia in the GBV-B-infected monkey with acute clearance. On the other hand, the rates of NK cells expressing the cytotoxic markers in the chronically infected monkey were as low as that in naïve and GBV-B-cured marmosets after acute phase, despite the fact that high viremia continued in the chronically infected monkey. These results suggest that the induction of antibodies and cytotoxicity of NK cells may be the host factors which associated with the clearance of GBV-B infection.

The kinetics of anti-E2, core and NS3 antibodies varied widely among chronically infected monkeys. Because the sequences of inoculated GBV-B are same, host factors may be associated with the timing of antibody induction and antibody titers. Recently it started to characterize major histocompatibility complex (MHC) class I and II in marmosets (191, 192). In order to analyze precise mechanisms that induce antibody, it would be necessary to compare the outcomes of GBV-B infection between marmoset groups which are divided by MHC in order to set genetic background. Regarding cellular immune response, the kinetics of CD335⁺ NK cells were able to be observed periodically in the acute phase of GBV-B infection. In this study, it is implied that CD335⁺ NK cells promote excluding GBV-B, which is consistent with the report that intrahepatic CD335^{high} NK cells was inversely correlated with HCV RNA levels (178)

and that expressions of CD335/NKp30 in patients who cleared HCV or who were uninfected with high HCV exposure were normal and high (184). In addition, previously it was reported that IFN γ was induced in the acute phase of GBV-B-infected marmosets (89). This T cells response was directed against GBV-B NS proteins and the response was associated with clearance of viremia. Taken together, both in HCV and GBV-B infection, cellular immune responses can be one of the factors which associated with the control of these infections.

For further study, I will analyze NAb titer against mutated GBV-B by using plasma collected periodically and GBV-B pseudo particles expressing GBV-B E1, E2 of which sequence are from mutated E1, E2. My colleague; Dr. Higashino investigated that mutations of GBV-B RNA from plasmas collected periodically in chronically infected monkeys by next-generation sequencing. , From this analysis, I can evaluate whether the mutations escaped from NAb and whether some GBV-B mutation sites were one of the factors of persisting infection or not.

These results suggest that the induction of antiviral responses including the antibodies and cytotoxic NK cells may be the host factors which restrict viral replication and persisting infection.

Chapter 4

**Attempt to improve a feasible non-human primate model
for evaluating hepatitis C vaccine**

4.1. Abstract

It has been shown that cellular immunity is crucial for controlling viral replication and limiting chronic infection. In fact, hepatitis C virus (HCV)-specific T cell response is exhausted in chronically HCV-infected individuals. In the chimpanzees transiently depleted of CD8⁺ T cells, virus replication was prolonged despite the presence of memory CD4⁺ T helper cells, indicating the critical role of CD8⁺ T cells for the viral control. In 3rd chapter, the inefficient induction of cellular immunity may involve in the persistent GB virus B (GBV-B) infection. The previous report also suggests that the clearance of GBV-B is associated with the appearance of IFN γ production. On the other hand, the rate of development of chronic GBV-B infection after acute infection is only 10% according to all accumulated data of GBV-B infected New World monkeys (NWMs) in my laboratory. I hypothesized that it may be common that the restriction factor(s) inhibit chronic GBV-B infection and the restriction factors(s) inhibit replication efficiency of HCV/GBV-B chimera. Taken together, cellular immunity may be a key to prolong HCV/GBV-B viremia. So I focused on CD8⁺ cytotoxic T cells (CD8⁺ T cells) and sought to decline cellular immunity in order to establish an animal model which shows high replication efficiency.

It was established that the protocols to deplete CD8⁺ T cells in chimpanzees and macaques, but not in NWMs. Recently Ito et al. established a marmosets CD8 specific monoclonal antibody (mAb, 6F10). In this study, I investigated whether the mAb is able to deplete CD8⁺ T cells transiently or not. After administration of the CD8 mAb, marmoset CD8⁺ T cells were efficiently depleted for three weeks or longer. In addition, other subsets, B cells and Natural Killer (NK) cells, were scarcely affected through this

experiment. I hope that this method will contribute to establish the animal model by which shows higher replication efficiency and virulence.

4.2. Introduction

It is also reported that cellular immunity, especially rapid and robust hepatitis C virus (HCV)-specific T cell response and the induction of intrahepatic IFN γ are associated with protective immunity (193). Although these observations strongly indicate an association between the timing, strongness, and specificity of the cellular immune response and the outcome of HCV infection, it is still unveiled that these T cell responses are the cause or the consequence of viral clearance. An actual role for T cell responses in HCV clearance was proven in the chimpanzee model by the finding that *in vivo* depletion of either CD4⁺ or CD8⁺ T cells prevents HCV clearance and clinical recovery (93-95). In CD8⁺ T cells depletion study, virus replication was prolonged despite the presence of memory CD4⁺ T helper cells in HCV-infected chimpanzees (93). These results suggest that CD8⁺ T cells are one of the essential lymphocytes to prevent long-term HCV infection. As well, in Old World monkeys the technique that *in vivo* depletion of CD8⁺ T cells by administration of a CD8-specific mAb has been established but not in New World monkeys (NWMs) (97-100). To decline cellular immunity of marmoset to establish animal model for higher replication efficiency, I sought to apply this technique to marmosets using the novel mAb 6F10 specific for marmoset CD8 (85). In this study, I demonstrated for the first time in NWMs that the administration of the 6F10 mAb efficiently depleted CD8⁺ T cells in common marmosets (*Callithrix jacchus*). This work was contributed equally with my colleague; Dr. Yoshida. He predominantly performed binding competition among anti-CD8 mAb. And I injected mAb into marmosets in cooperation with him. I predominantly analyzed the *in vivo* depletion of CD8⁺ T cells in marmosets.

4.3. Materials and Methods

Animals

A total of three common marmosets, weighing 357-457 g, reared at the Primate Research Institute, were used for the study of *in vivo* depletion of CD8⁺ lymphocytes. All these animal experiments were conducted in accordance with the protocols of experimental procedures that were approved by the Animal Welfare and Animal Care Committees of the Primate Research Institute of Kyoto University, Japan. All animals were caged individually at 27 ± 2°C in 50 ± 10% humidity with a 12 h light-dark cycle (lighting from 7:00 to 19:00). All animals were fed twice a day with a standard marmoset diet supplemented with fruit and mealworm. Water was given *ad libitum*. Marmoset blood were collected under anesthesia (ketamine, <10 mg/head, intramuscularly).

Flow cytometry

Flow cytometry was performed as previously described with a slight modification (188). For analysis of CD8⁺ T cells depletion, a previously established mouse anti-marmoset CD8 mAb named 6F10 was used (85). The 6F10 mAb was conjugated with allophycocyanin (APC) and by Zenon Mouse IgG labeling Kit (Molecular Probes) according to the manufacturer's instruction. Fifty microliters of whole blood from marmosets was stained with combinations of fluorescence-conjugated mAb: APC-Cy7-conjugated anti-CD3 (SP34-2: Becton Dickinson), PerCP-Cy5.5-conjugated anti-CD4 (L200: BD Pharmingen), PE-conjugated anti-CD8 (CLB-T8/4, 4H8 (CLB hereafter): Sanquin; RPA-T8 (T8 hereafter): Becton Dickinson) and FITC-conjugated

anti-CD20 (H299; BECKMAN COULTER). Then, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After washing with a sample buffer containing phosphate buffered saline (PBS) and 1% fetal bovine serum (FBS), the labeled cells were resuspended in a fix buffer containing PBS and 1% formaldehyde. The expression of the immunolabeled molecules on the lymphocytes was analyzed with a FACSCanto II flow cytometer (Becton Dickinson). The data analysis was conducted using FlowJo software (Treestar, Inc.).

***In vitro* binding competition of anti-CD8 mAbs**

Fifty microliters of whole blood from marmosets was treated with increasing amounts (1, 10, 100 ng) of the 6F10 mAb on ice for 30 min. After washing with the sample buffer, the cells were stained with fluorescence-conjugated mAbs against CD3, CD4, and CD8 on ice for 30 min. Then, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After washing with the sample buffer, the labeled cells were resuspended in the fix buffer. The fluorescence intensity of the cells was analyzed as described above.

***In vivo* depletion of CD8⁺ T cells**

In vivo depletion of the marmoset CD8⁺ T cells was performed as previously described (100). Briefly, the 6F10 or a control mAb (MOPC-21) was administrated subcutaneously to the subject at 10 mg/kg (body weight) followed by intravenous administration at 5 mg/kg in the saphenous vein at a rate of 20 ml/h using a syringe pump on days 3, 7, 10 after the primary administration. The kinetics for the percentages of CD8⁺ and CD4⁺ cells in a CD3⁺ T cell subset as well as the percentages of CD20⁺ B

cells and CD3⁺ CD20⁺ cells in the total lymphocytes of each marmoset were periodically monitored during the observation period as indicated.

Statistical analyses

Statistical analyses of lymphocyte ratios were performed using Student's *t*-test and single-factor ANOVA, followed by Fisher's protected least-significant difference *post hoc* test by using StatView software (SAS Institute).

4.4. Results

Lymphocyte subsets in marmosets

Ito et al. reported that 6F10 mAb specifically detected CD8⁺ T cells in marmosets by using flow cytometry as well as immunohistochemical and Western blot analyses (85). Basic information regarding flow cytometric analyses of CD4/CD8 naïve and central/effector memory T cells and Natural Killer (NK)/NKT cells in marmosets was available from the recent report (92). Dr. Yoshida and I compared the immunoreactivity of the 6F10 mAb with other commercially available CD8 mAbs in lymphocyte subsets of marmosets (Fig. 16). The gating strategy for profiling CD4⁺ and CD8⁺ T cells was shown in Fig 16. The percentage of CD8⁺ T cells in a CD3⁺ T cell subset as detected by 6F10, CLB or T8 anti-CD8 mAb was comparable (23.5%, 23.2% and 22.9%, respectively). Notably, the 6F10 mAb poorly cross-reacted with tamarin and rhesus macaque CD8⁺ T cells, while CLB and T8 mAbs did with both (data not shown). It is reasonable that the 6F10 mAb showed selected specificity to the marmoset CD8, considering that it was established by immunization with marmoset lymphocytes (85).

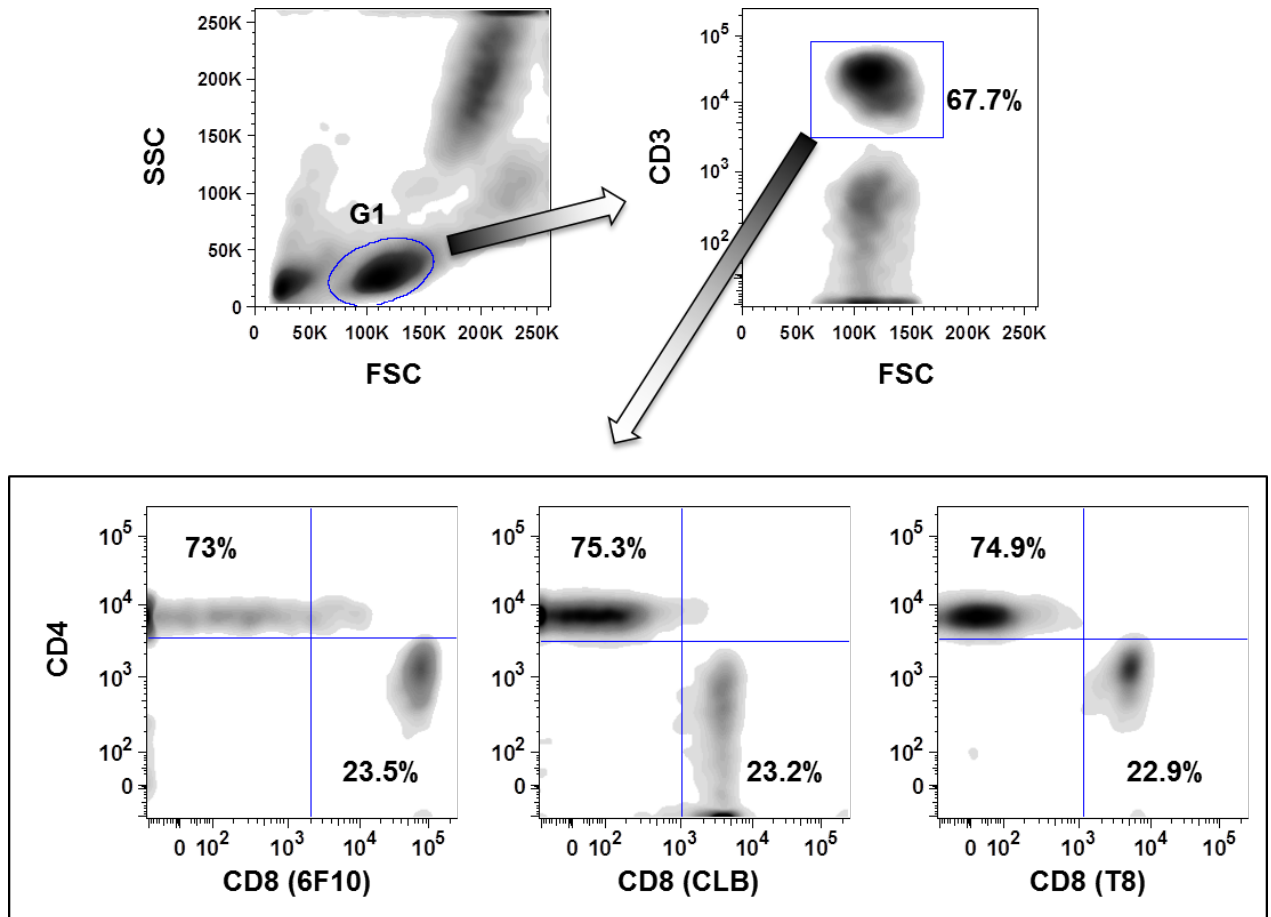


Fig. 16 Flow cytometric analyses of CD3, CD4 and CD8 expressions on lymphocytes in marmosets

Fifty microliters of whole blood specimens from marmosets were stained with APC-Cy7-conjugated anti-CD3, PerCP-Cy5.5-conjugated anti-CD4 and APC-conjugated 6F10 mAbs or PE-conjugated CD8 (CLB or T8) mAb. Then, erythrocytes were lysed and the stained cells were resuspended in the fix buffer. Representative results in a marmoset are shown. The G1 lymphocyte population was selected (left top panel) and a CD3⁺ T cell subset was gated (right top panel). Fluorescence intensity for CD4⁺ and CD8⁺ in the T cell subsets was depicted (lower panels).

***In vitro* binding competition of anti-CD8 antibodies in CD8⁺ T cells of marmosets**

We initially treated the marmoset lymphocytes with increasing amounts of APC-conjugated 6F10 together with fluorescence-labeled mAbs to CD3 and CD4. It was found that fluorescence intensity for APC on a CD3⁺ T cell subset was saturated by 10 ng or more of 6F10 mAb (Fig. 17a). We then sought to define whether the binding epitope of 6F10 mAb in the marmoset CD8 molecule was overlapped with the epitopes of T8 and CLB mAbs by a competition assay. It was found that fluorescence intensity in the CD3⁺ T cell subset treated with the labeled CLB mAb was drastically reduced by the pretreatment of 10 or 100 ng 6F10. On the other hand, the 6F10 pretreatment scarcely influenced fluorescence intensity in the cells that reacted with the labeled T8 mAb, irrespective of the amounts of 6F10 (Fig. 17b, c). These results indicated that 6F10 competitively inhibited binding of CLB but not T8 mAbs to CD8, suggesting that the binding epitope for 6F10 was overlapped with that of CLB and was sterically apart from that of T8. In addition, T8 was likely to exhibit greater affinity than CLB to marmoset CD8 (Fig. 17b).

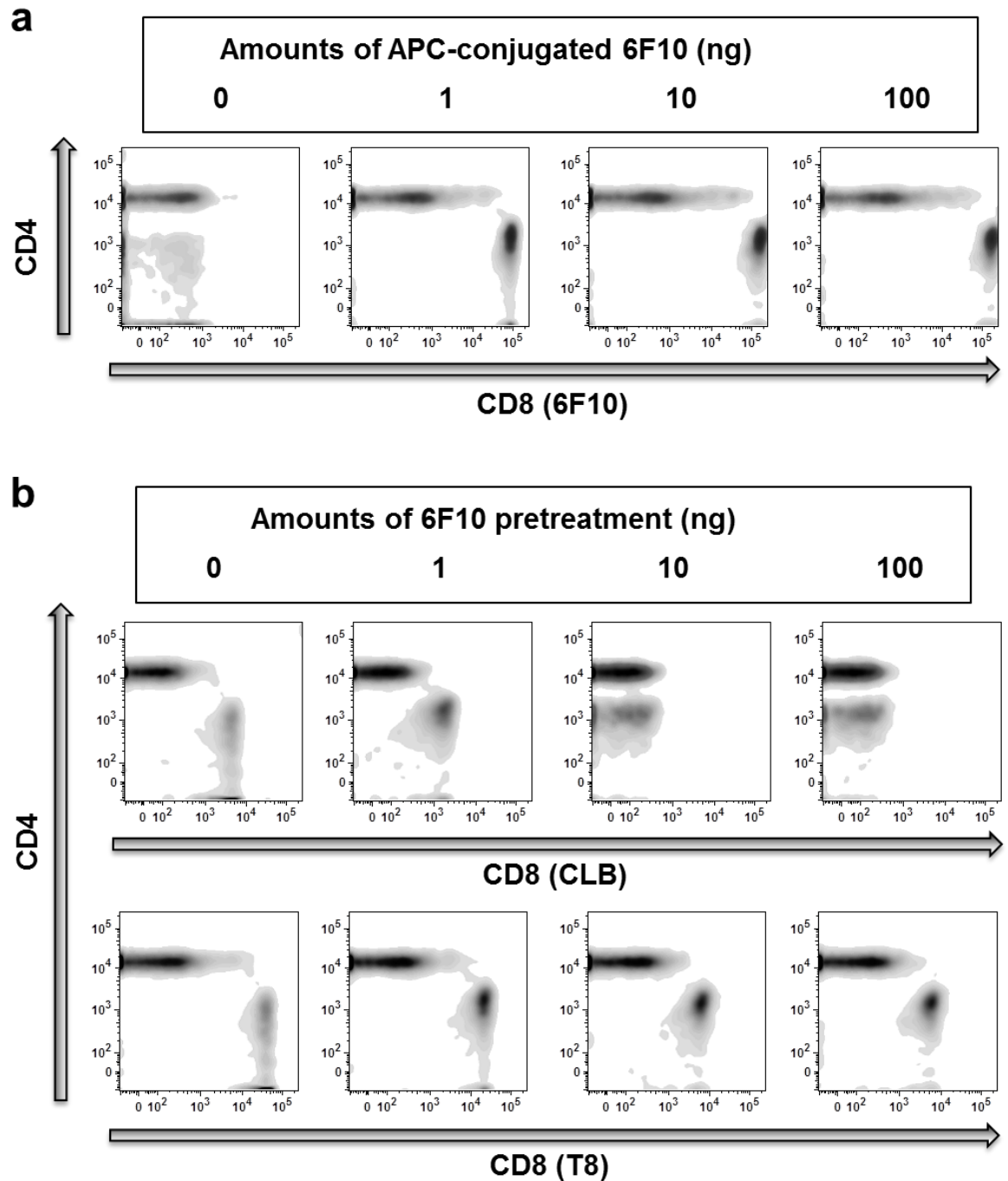


Fig. 17 Binding competition among anti-CD8 mAbs in marmoset CD8⁺ T cells

(a) Fifty microliters of whole blood specimens from marmosets were stained with APC-Cy7-conjugated CD3 and PerCP-Cy5.5-conjugated CD4 mAbs and increasing amounts (1, 10, 100 ng) of APC-conjugated 6F10 mAb. The fluorescence intensity for CD4⁺ and CD8⁺ cells in CD3⁺ T cell subsets were shown.

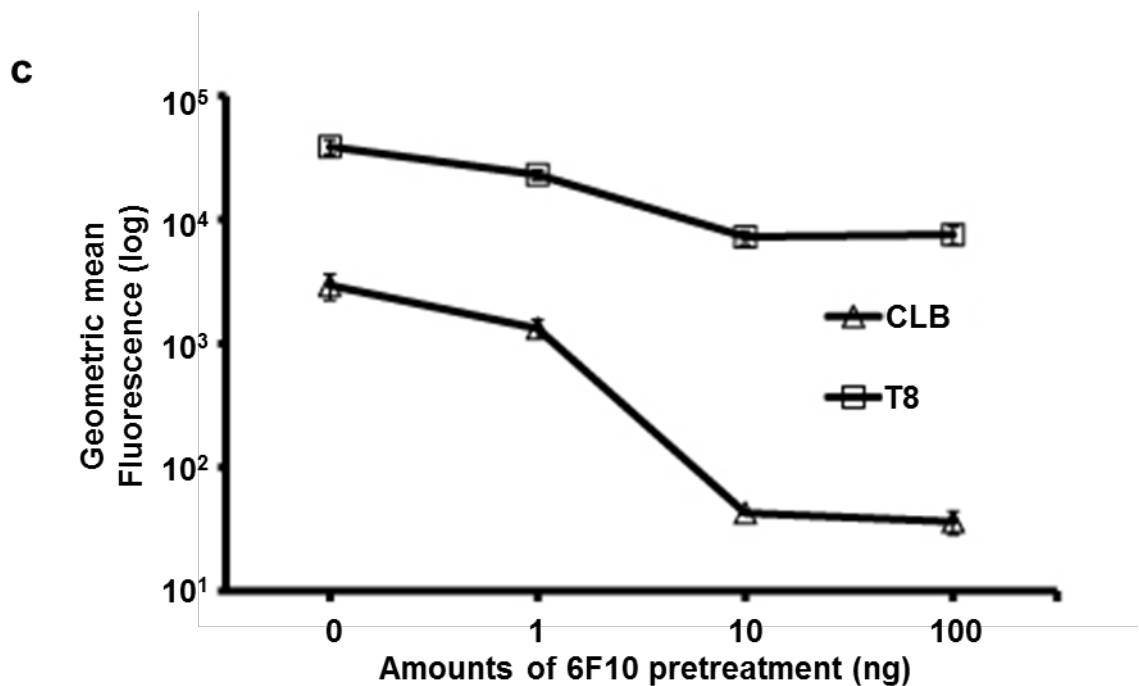


Fig. 17 (continue)

(b, c) Fifty microliters of whole blood specimens were pretreated with increasing amounts (1, 10, 100 ng) of 6F10 mAb, followed by staining with fluorescence-conjugated mAbs against CD3, CD4, and CD8 (CLB or T8). Fluorescence intensity of CD4⁺ and CD8⁺ cells in CD3⁺ T cell subsets were shown (b) and the geometric mean fluorescence of CD8⁺ T cells labeled by the CLB or T8 mAbs were indicated (c). I analyzed statistically whether geometric means were different in each antibody by using StatView software.

***In vivo* depletion of CD8⁺ T cells using an anti-marmoset CD8 mAb**

I finally examined whether the administration of the 6F10 mAb could influence CD8⁺ T cells *in vivo*. Three marmosets were subcutaneously administrated at 10 mg/kg followed by intravenous administration at 5 mg/kg on days 3, 7, 10 after the primary administration. The mAb-treated marmosets did not develop any clinical and hematological signs (data not shown). In order to detect CD8⁺ T cells in the 6F10-treated marmosets, I employed T8 mAb, which was found to react with CD8 molecule in the presence of 6F10 as shown in Fig. 17. It was found that at ten days after the mAb administration the CD8⁺ T cells were almost completely depleted, followed by gradual recovery to a half of the initial levels at around 4-7 weeks later (Fig. 18). It is noteworthy that the treatment relatively increased in the proportion of CD4⁺ T cells in compensation for the depletion of CD8⁺ T cells, while the ratios of CD20⁺ B cells and CD3⁻ CD20⁻ cells were scarcely affected (Fig. 19). In addition, administration of a control antibody (MOPC-21) did not affect any lymphocyte subsets (data not shown). These results demonstrated that the 6F10 mAb was able to specifically deplete CD8⁺ T cells in marmosets.

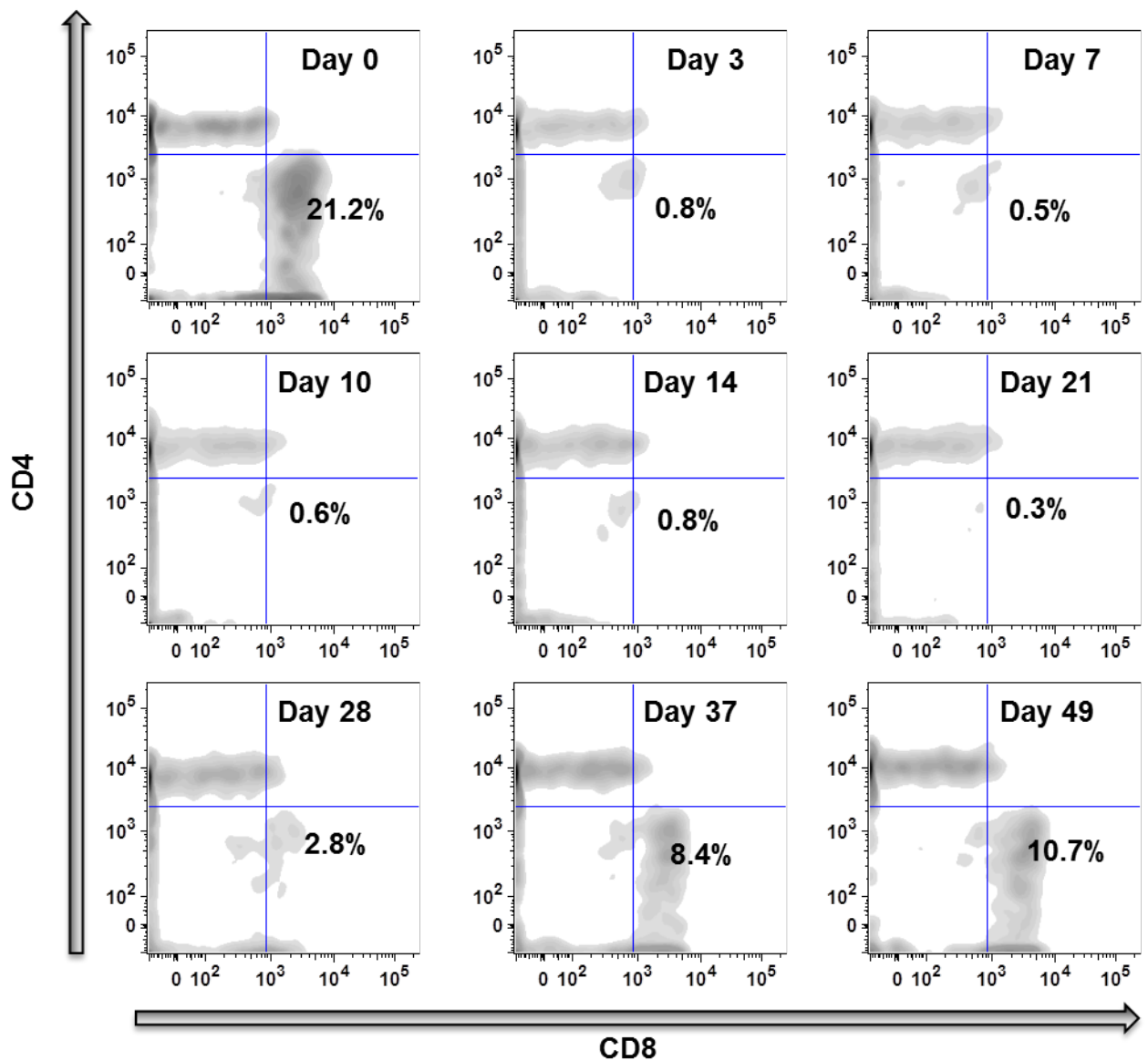


Fig. 18 *In vivo* depletion of CD8⁺ T cells by administration of a marmoset anti-CD8 mAb 6F10

Periodical kinetics for the fluorescence intensity of CD4⁺ and CD8⁺ cells in a CD3⁺ T cell subsets of a marmosets (Cj175) administered subcutaneously with 10 mg/kg of the 6F10 mAb, followed by 5 mg/kg administration intravenously at days 3, 7, and 10 were shown.

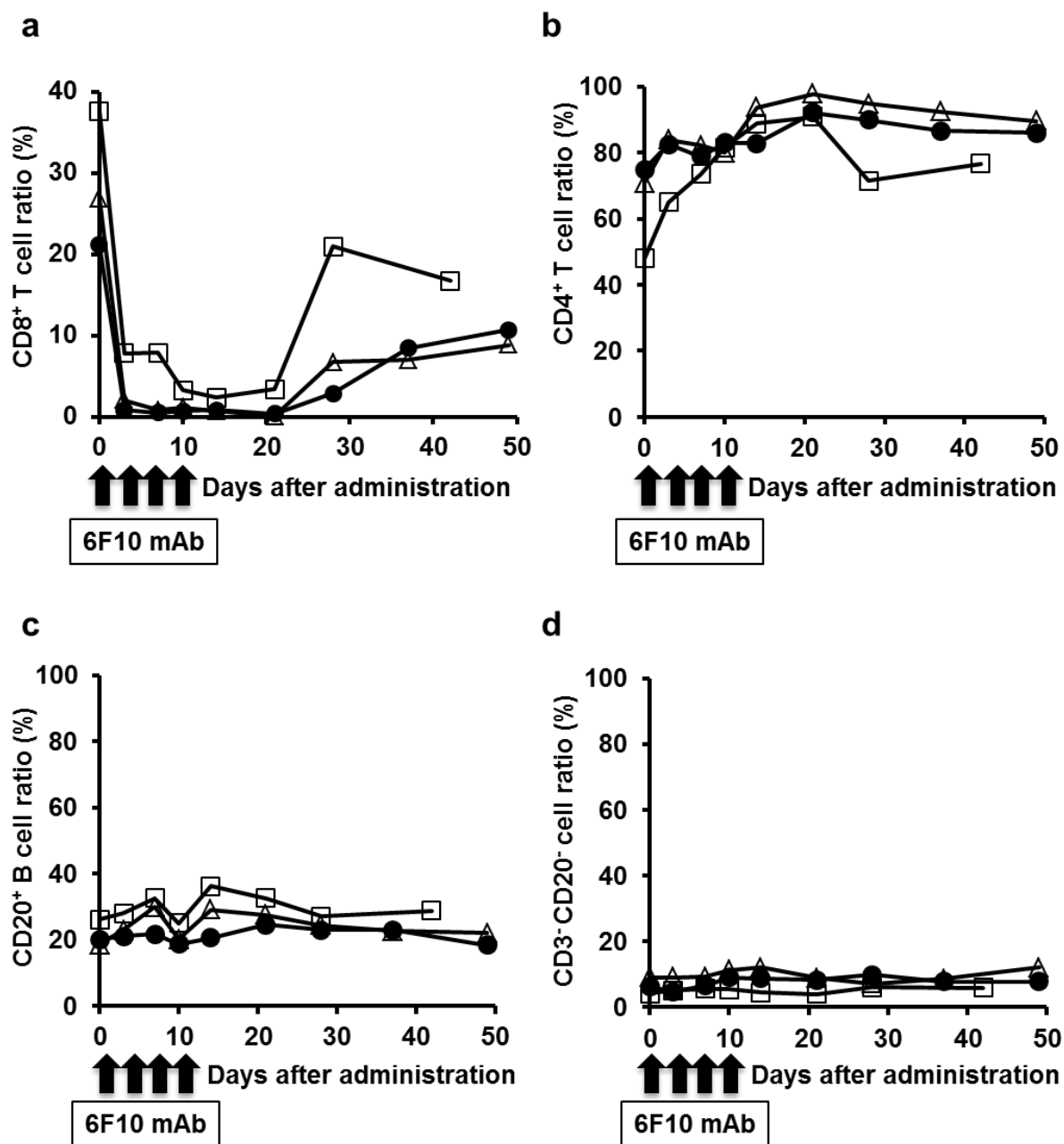


Fig. 19 The kinetics of lymphocytes in three marmosets administrated CD8 mAb

The kinetics of CD8⁺ cells (a) and CD4⁺ cells (b) in a CD3⁺ T cell subsets as well as CD20⁺ B cells (c) and CD3⁻ CD20⁻ cells (d) in total lymphocytes of each marmoset after the administration of the 6F10 mAb were shown.

4.5. Discussion

In order to apply new insights found in 3rd chapter to an animal model in which chimera replicates efficiently, I sought to decline the cellular immunity by depleting cytotoxic T cells (CD8⁺ T cells) in marmosets. And I attempted to establish a technical basis for the study of CD8⁺ T cells in marmosets. I assessed the effect of a 6F10 mAb administration *in vivo* and found that CD8⁺ T cells were efficiently depleted in the blood of the treated monkeys for three weeks or longer and that in compensation for the depletion, the proportion of CD4⁺ T cells were relatively increased without obvious influence on other lymphocyte subsets such as CD20⁺ B cells and CD3⁺ CD20⁻ cells. This is the first report showing the establishment of new methodology to deplete common marmoset CD8⁺ T cells *in vivo*. Since demand for marmosets as non-human primate models for a variety of inflammatory and autoimmune diseases as well as the hepatitis C vaccine study has been increasing, my findings will provide new techniques to scientists who are eager to examine the pivotal role of CD8⁺ T cells *in vivo* in the onset of the human diseases.

Common marmosets are suitable for detailed observations of the movement of the extremities and cognitive functions, which approximate those of humans. Therefore, marmosets are highly useful as models of neurological diseases such as Parkinson's, MS and RA. Importantly, the marmoset models for analyzing Parkinson's disease and autoimmune diseases such as MS and RA have already been developed (86-88, 194). CD8⁺ T cells have been implicated in the pathogenesis of autoimmune disorders including diseases of the central nervous system such as MS, encephalomyelitis and diabetes mellitus (195-197). MS is an immune-mediated disease of the central nervous system leading to demyelination and axonal/neuronal loss. Accumulating evidence

points to a key role for CD8⁺ T cells in the diseases; histopathological analyses and compelling observations from animal models indicate that cytotoxic CD8⁺ T cells target neural cell populations with the potential of causing lesions consistent with MS (198). RA is a systemic and chronic autoimmune disease characterized mainly by synovial inflammation leading to joint destruction and disability with a huge impact upon the quality of life and life expectancy. Several studies have demonstrated that CD8⁺ T cells in RA have powerful cytotoxic ability and therefore exhibit the potential to enhance the disease (199). Thus, my *in vivo* CD8 depletion technics will be valuable in other examining the role of CD8⁺ T cells in these autoimmune diseases not only evaluation of hepatitis C vaccine candidates in the marmoset models.

To evaluate animal model in which GBV-B replicates efficiently, I am planning to inoculate GBV-B into CD8⁺ T cells depleted marmosets and examine whether they would develop chronic infection. In addition, *in vivo* depletion of CD16⁺ NK cells in tamarins has already established (188), and the technique can be applied to marmosets. Depletion of the CD16⁺ NK cells by a mouse anti-human CD16 (3G8) mAb or in combination with the mAb against CD8⁺ T cells in NWMs may be promising to induce virus replication efficiently since I showed in 3rd chapter that NK cells may contribute to the viral control. Furthermore, one of the factors of chronic infection is antibody response, and thus depletion of B cells can also be considered. *In vivo* depletion of CD20⁺ B cells in marmoset preclinical MS model and experimental autoimmune encephalomyelitis model has previously been established (194, 200, 201). CD20⁺ cells were depleted by human anti-human CD20 IgG1 κ mAb (HuMab 7D8) that cross-reacts with marmoset CD20. Thus it is theoretically feasible to deplete in combination both cellular and humoral immune responses, which would accelerate chronic GBV-B

infection in NWMs, although side effects including immunocompromised diseases must be considered.

General Discussion and Conclusion

In my study, I sought to establish an animal model for evaluation of hepatitis C vaccine candidates to accomplish hepatitis C vaccines which are available around the world. In order to improve the animal model in which hepatitis virus replicates efficiently, I focused on the host factors which showed the differences between acute and chronic GBV-B infection and analyzed their implication in the persistent GBV-B infection. Then, I established the method which has the ability to improve the chimera-infected NWMs model for the first time.

In 1st chapter, I inoculated the novel hepatitis C vaccine candidate with adjuvants into marmosets and evaluated the safety of the vaccine candidate. The vaccine candidate was established by my collaborators; Dr. Kato and his colleagues in Department of Virology II, National Institute of Infectious Diseases, composed of inactivated whole-virion prepared from HCVcc. As a result, I found that the combination of inactivated HCVcc and K3-SPG induced effective cellular immune response against HCV antigen without any obvious side effects. Together with the data in terms of antibody responses provided by the collaborators, I concluded that the inactivated HCVcc and K3-SPG may be promising as a novel hepatitis C vaccine candidate. However, in order to evaluate the prophylactic and therapeutic effects of this vaccine candidate, it is necessary to develop an animal model considering the present situation that no feasible animal model is available to evaluate the prophylactic and therapeutic effects of the hepatitis C vaccine candidate. In 2nd chapter, for the purpose of developing a system which can evaluate vaccine candidates, I thus sought to establish a novel HCV/GBV-B chimera of which majority of the viral genome was derived from

HCV and evaluated the replication ability *in vitro* and *in vivo*. I found that the HCV/GBV-B successfully replicated in a tamarin and moreover the chimera exhibited persistent replication for more than two years after challenge. This is the first finding that HCV-based chimera persistently replicated *in vivo*. The chimera-infected NWMs can be a promising animal model, but still replication efficiency and virulence should be improved. In 3rd chapter, aiming at improving replication efficiency, I focused on the host factors and compared the humoral and cellular immune responses between acute and chronic phases of GBV-B infection. In chronically infected monkeys, antibody responses against GBV-B core, E2 and NS3 were much delayed than that in monkeys with acute clearance. In addition, NK cells expressing cytotoxic markers increased after viremia in the GBV-B-infected monkey with acute clearance. In contrast, chronically infected monkeys showed low expression rates of the cell population expressing cytotoxicity markers as much as that in naïve and the GBV-B-cured monkeys. I concluded that the induction of antibodies and cytotoxicity of NK cells may contribute to the establishment of persistent infection. To apply these findings to the animal model, I sought to examine if the cellular immunity could be artificially controlled by depleting CTL (CD8⁺ T cells) in marmosets. The collaborator; Dr. Yoshida and I depleted CD8⁺ T cells by administration of a novel CD8-specific mAb in NWMs for the first time and could establish the methodology that depleting CD8⁺ T cells drastically and specifically. I hope I will apply this method to make GBV-B or HCV/GBV-B chimera infects persistently and efficiently in NWMs.

In the view of methodology of hepatitis C vaccine, I will consider the method of prime-boost vaccination because several features of the vectors used in prime-boost vaccination may underlie their capacity to enhance immune response, particularly

cellular immunity (202). Prime-boost vaccination is an effective vaccination with high immunological potency, using different kinds of vector like DNA vaccine and attenuated recombinant viral vectors such as fowlpoxvirus (FPV) and modified vaccinia virus Ankara strain (MVA), in which express same target antigens (111, 202). In phase I clinical study of hepatitis C vaccine candidates by using the prime-boost vaccination, replication defective adenoviral vector and MVA vector encoding the NS3, NS4, NS5A and NS5B proteins of HCV genotype 1b generated very high levels of CD8⁺ and CD4⁺ HCV-specific T cells targeting multiple HCV antigens (203). Sustained memory and effector T cell populations are also generated. Thus, the combination of the inactivated HCVcc with a viral vector encoding some parts of HCV protein may be promising as a prime-boost vaccination in order to induce durable, broad and sustained T cell responses against HCV in further study.

HCV/GBV-B chimera must replicate stably in an animal model that is capable of evaluating prophylactic and therapeutic effects of hepatitis C vaccine candidates. To gain an animal model which shows higher and persistent replication efficiency of the virus, it is beneficial that HCV/GBV-B chimera is passaged through marmosets for the purpose of *in vivo* adaptation of the virus. In the case of HIV research, it is already reported that simian-human immunodeficiency virus (SHIV) which is chimera virus of HIV and simian immunodeficiency virus (SIV), replicates more efficiently after some passages in macaques (204, 205). SHIV chimera which inoculated into pigtailed macaques that were transiently depleted of CD8⁺ T cells, acquired the ability to antagonize the macaque restriction factor, replicated at progressively higher levels and ultimately caused acquired immune deficiency syndrome (AIDS)-defining conditions (205). As well as HIV study, it is already reported that HCV is passaged through

chimpanzees which are not depleted of CD8⁺ T cells. The courses of HCV infection were similar between 1st and 2nd passages, both caused acute hepatitis and some chimpanzees caused chronic infection (206). So I expect that HCV/GBV-B chimera will also replicate more efficiently in marmosets which are depleted CD8⁺ T cells after some passages. And then, some mutations in the chimera may induce adaptation to replicate with higher rate also in normal marmosets which are not depleted of CD8⁺ T cells. Once the ideal animal model, a combination of the monkey-adapted HCV/GBV-B chimera and normal marmosets, is established, the adapted HCV/GBV-B chimera will be inoculated into normal marmosets after administrating a prophylactic vaccine candidate, by which I may evaluate the prophylactic effect of the candidate by checking the replication level of the chimera. In addition, after the adapted HCV/GBV-B chimera replicates and causes hepatitis in normal marmosets, a therapeutic vaccine candidate is administered then can be evaluated the therapeutic effect of the candidate by checking if the chimera would be excluded or hepatitis could be alleviated. Even if I would not establish the animal model which shows higher replication efficiency in normal marmosets which are not depleted CD8⁺ T cells after some passages, the animal model which is depleted CD8⁺ T cells can evaluate some vaccine candidates which induce humoral immune response against HCV. Evaluation of vaccine candidates by using these animal models as a preclinical study facilitates selection of vaccine candidates before clinical human study. Finally I hope that effective prophylactic/therapeutic hepatitis C vaccines will be approved and will contribute to saving people from hepatitis C.

My data of HCV/GB chimera constructed by GBV-B structural proteins was not consistent with Li et al.'s data of chimeras constructed by HCV structural proteins (176).

The chimeras established by Li et al. caused persistent infection in a marmoset for 43 weeks after inoculation. Another group reported that HCVpp efficiently infected tamarin hepatic cell line and primary marmoset hepatocytes, while HCV/GBV-B chimera which is constructed by GBV-B structural proteins, p6p7 and NS2 failed to induce viremia in a tamarin after intrahepatically inoculation (207). These conflicts might be explained by some different situations such as the strains of HCV, substitution sites and the amount of inoculated virus RNA. In addition, it is possible that host factors restrict host tropisms of HCV and GBV-B as well as viral construct. Since NWMs experimented in HCV study are in the diverse genetic backgrounds unlike inbred strain of mice, the dispersion of results may occur easily in NWMs. The genetic backgrounds which affect the outcomes of HCV or GBV-B infection may be related to type III IFN (IFN- λ) single nucleotide polymorphism (SNP). SNP is a variation at a single position in DNA sequence among individuals. SNP is defined as more than 1% of a population does not carry the same nucleotide at a specific position in the DNA sequence. In 2009, a group analyzed that two SNPs (rs8099917 and rs12980275) near the *IFNL* on chromosome 19 were strongly associated with SVR after pegIFN/ribavirin combination treatment (208). The frequencies of minor alleles-positive patients were much higher in null viral responder group than SVR group in both SNPs. Recently SNPs at several sites were found (209). Especially, a dinucleotide variant ss469415590 (TT or Δ G) on upstream of *IFNL3*, which is in high linkage disequilibrium with rs12979860, a genetic marker strongly associated with HCV clearance (210). The major allele TT causes a frameshift and disrupts the *IFNL4* ORF, while minor allele Δ G enables IFN- λ 4 protein expression. Interestingly, the major allele TT correlates with a favorable outcome of HCV infection. *IFNL4* mRNA was exclusively detected in specimens from patients with

chronic HCV infection. Therefore the activation of the *IFNL4* gene was found to be related to chronic HCV infection. In addition, compared to rs12979860, ss469415590 is more strongly associated with HCV clearance in individuals of African ancestry, although it provides comparable information in Europeans and Asians. Taking this information obtained from human study into consideration, it is possible that SNPs at the *IFN* coding region among marmosets or tamarins exist and critically affect outcome of GBV-B or HCV/GBV-B infection. In my laboratory, some mutations in the region of *IFNL3* between acute and chronic GBV-B infected marmosets were already found. Further analysis is ongoing.

In conclusion, I clarified four critical points in the aim of establishing an animal model for evaluating hepatitis C vaccines. The first is the effective hepatitis C vaccine candidate, the second is persistently replicated HCV-based chimera, the third is the mechanisms of chronic GBV-B infection, the forth is the method to decline CTL in NWMs. The final goal of my study is to develop hepatitis C vaccines by using the established animal model and to prevent prevalence of HCV infection by the broadly available hepatitis C vaccines. I believe that these knowledges found in my study are newly and critical for hepatitis C vaccine study and will contribute to control HCV and to effectuate the breakthrough of hepatitis C study in the future.

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