ることができない．Dectin-1による自然免疫応答活性化はTh1やTh17型の獲得免疫を誘導することができ，CTL反応をも誘導することが報告されている[5]．Th17型免疫応答は真菌感染防御に重要であることが明らかとされており，Dectin-1による自然免疫応答が重要な役割を担っていると考えられる．

Dectin-2（ヒト；*CLEC6A*，マウス：*Clec4n*）は樹状細胞やマクロファージなどに発現しており，真菌の細胞壁成分であるα-マンナンを認識することが知られている[8]．Dectin-2は細胞内シグナル伝達モチーフを欠損しており，ITAMモチーフを有するFcRγと結合することにより，自然免疫応答を活性化する．Dectin-2は真菌に対して，Th17型の免疫応答を誘導することが明らかとなっているが，アレルギー反応をも惹起することが報告されている．ハウスダストアレルギーの原因であるチリダニはDectin-2に認識されることで，Sykを介し，システイニルロイコトリエンの産生を誘導する[9]．これら脂質性メディエーターは好酸球性または好中球性の肺の炎症を誘導し，Th2型のアレルギー反応を促進する[10]．しかしながら，これらの獲得免疫反応をどのようにしてDectin-2が制御しているか，詳細は明らかとなっていない．

Mincle（macrophage-inducible C-type lectin）（CLEC4E）はマクロファージや好中球に低レベルで発現しているが，TLRsアゴニスト刺激によって，強く発現が上昇される[11]．Mincleはα-マンノースを含んだ真菌を認識することが知られている[12]．一方で，結核菌感染にも関与していることが報告され，結核菌細胞壁成分である，トレハロースジマイコレート（TDM）を認識することが明らかとされた[13]．TDMはコンプリートフロイントアジュバントの活性成分としても知られており，実際に結核菌のコンポーネントワクチンとともに投与することで，強いアジュバント活性（Th17型免疫応答の誘導）を示している[14]．

TLRsとC-typeレクチン

TLRsの同定後，個々の自然免疫受容体のシグナル伝達に注目して研究が行われてきた．その結果として，新たにいくつものTLRs以外の受容体が同定され，同時に詳細なメカニズムが明らかとなってきた．一方で，同じ病原体をいくつかの異なる自然免疫受容体が認識することも報告されており，お互いの自然免疫応答との関係には不明な点が多く残されていた．これまでに，いくつかのC-typeレクチンがTLRsを介した自然免疫応答を増強または，抑制することが報告されている．ここでは，TLRsとC-typeレクチンに着目し，自然免疫応答の制御に関して解説する．

β-グルカンの受容体であるDectin-1はTLRsとの関連が多く報告されている．実際に，ヒトの末梢血単核球をβ-グルカンの一種であるCurdlanとともにTLR2またはTLR4のリガンドであるPam$_3$CSK$_4$，LPSで刺激をすることにより，相乗的にTNF-αやIL-10の産生が増強された[15]．同様の結果がマウスのマクロファージを用いた実験によっても明らかとなっており，TLR2, 4だけではなく，TLR5やTLR7, TLR9のリガンド刺激においても，β-グルカンで細胞を同時刺激することでTNF-α産生の増強が確認された[16]．また，マウスのマクロファージを出芽酵母由来のβ-グルカンとPam$_3$CSK$_4$で同時刺激することで，IL-12 p70の産生の抑制が確認されている[17]．これは，β-グルカン刺激によって，IL-12 p70のサブユニットであるp35の産生が抑制されていることが原因となっているが，詳細なメカニズムは明らかとなっていない．p35の産生が抑制されていると同時に，IL-6やIL-23の産生がβ-グルカンとPam$_3$CSK$_4$共刺激によって促進されていることも報告されている[17]．IL-6やIL-23はT$_H$17細胞の誘導に必須なサイトカインであり，TLRs, CLRs両方の自然免疫シグナルが活性化されることで，強くTh17型獲得免疫応答を誘導していると考えられる[18]（図2）．実際に，真菌感染時にはCLRsのみならずTLRsも認識に関与しているために，真菌を効率よく排除するために異なる自然免疫受容体が存在していると考えられる．また，TLR9のリガンドであるCpG ODNと真菌のβ-グルカンの強刺激によって，IL-12 p70の産生がDectin-1依存的に抑制されていることも示されている[19]．同様に，真菌由来の多糖がTLR9を介したIL-12 p40の産生を抑制することも報告されている[20]．最近の報告で，Dectin-1シグナルがサイトカインシグナル抑制因子（SOCS1）の発現を促進することが報告された[21]．SOCS1はその名のとおり，サイトカイン受容体か

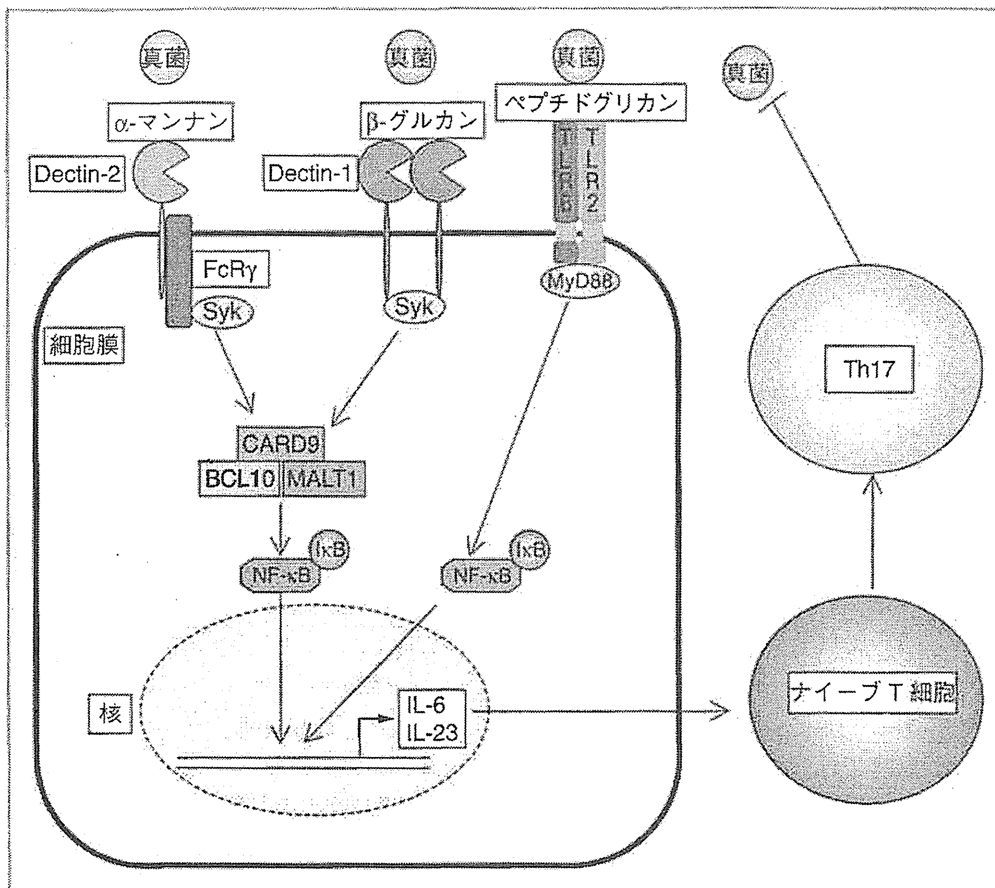

図2 CLRsとTLRsによる自然免疫と獲得免疫の誘導

らのシグナル伝達を抑制する機能を有しており，TLR9アゴニスト刺激によるIL-12やIL-10の産生をも抑制することが明らかとされた[21]．われわれも，まだ未発表ながらTLR9によるⅠ型IFN産生がDectin-1シグナルによって，抑制されることを明らかとしている．これらの結果から，TLRsとDectin-1は真菌に対する自然免疫受容体として共同して働き，TLRsリガンドによるTh1型の免疫応答をDectin-1シグナルによってTh17型にシフトさせていることが考えられる．

Blood dendritic cell antigen 2（BDCA-2，CD303）はヒトの形質細胞様樹状細胞（pDCs）に特異的に発現しているC-typeレクチンである．BDCA-2に対する抗体を用いて，BDCA-2を介したシグナル伝達を活性化することで，強く樹状細胞からのⅠ型IFN産生が抑制されることが報告されている[22]．また，IFN遺伝子のmRNAレベルでの低下も確認されていることから，BDCA-2シグナルが少なくとも転写の前に抑制的に働いていることを示唆している[23]．これらのことから，BDCA-2に対する抗体は，Ⅰ型IFNの産生亢進が病因に関与している全身性エリテマトーデスなどの自己免疫疾患患者に対して，Ⅰ型IFN産生を調節するための抗体医薬としても注目されている．

Dendritic cell immunoreceptor（DCIR）は単球やマクロファージ，樹状細胞での発現が確認されており，マンノースやフコースを基本とした多糖を認識することが知られている．ヒトDCIRもBDCA-2と同様の機能が報告されており，DCIRシグナル活性化によって，TLR8やTLR9リガンド刺激によるⅠ型IFN産生を抑制することが報告されている[24][25]．実際に，TLR9リガンド刺激によって，BDCA-2やDCIRの発現が低下していることも明らかとなっており，これらのシグナル伝達はお互いに制御し合っていると考えられる．一方で，BDCA-2とDCIRはHIV-1と結合することが報告されており，実際にHIV-1のgp120はBDCA-2と結合することにより，pDCからのIFN-α産生を

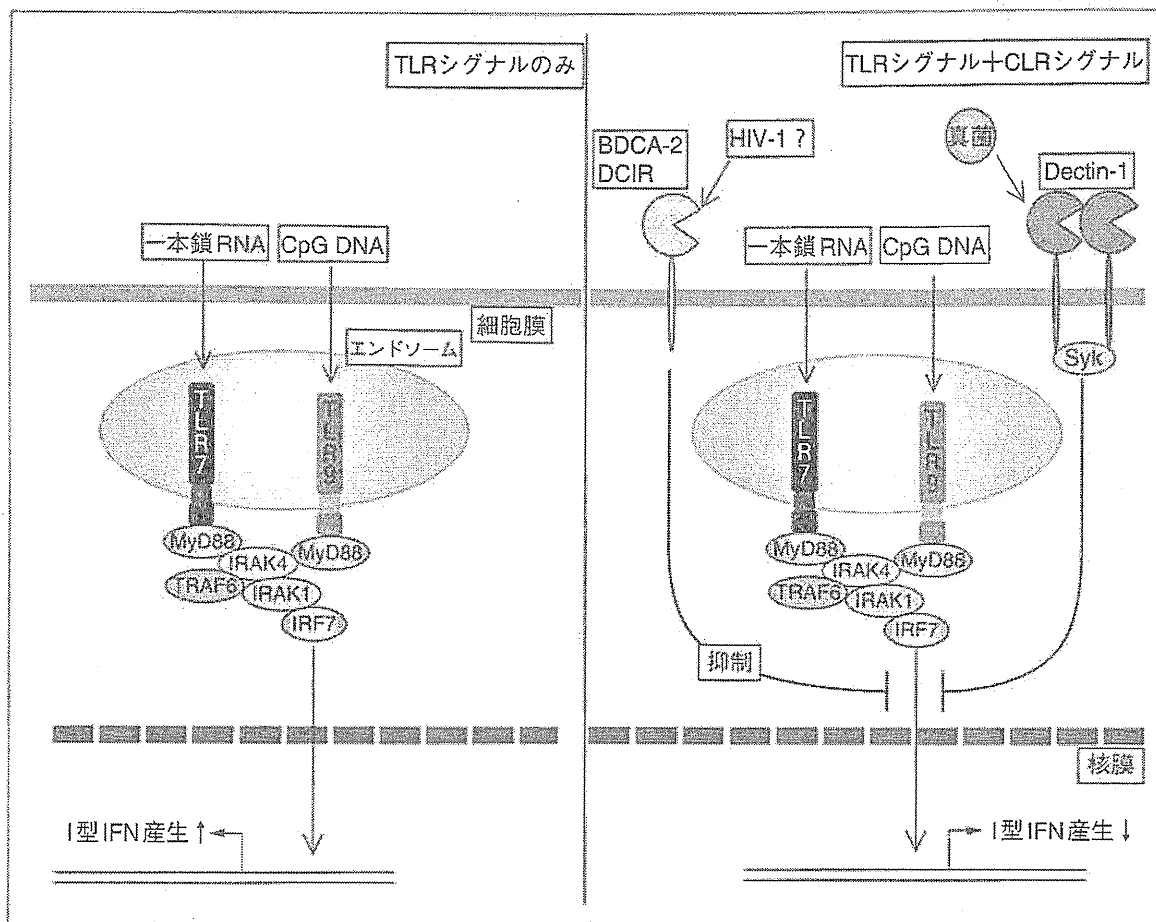

図3 CLRsシグナルによるTLRsシグナルの抑制

抑制することが報告されている[26)27)]．これらの結果は，BDCA-1とDCIRはpDC上に発現し，TLRsリガンド刺激による自然免疫応答を抑制的に働いていることを示唆している(図3)．しかしながら，HIV-1感染や排除との関連など，不明な点は多く残されており，HIV-1の病態解明のためにも今後の研究成果が待たれる．

CLRsであるDEC-205(CD205)は，マウスでは主に樹状細胞やT細胞，B細胞にも発現しており，エンドサイトーシスなどに関与していることが考えられていたが，その機能に関しては不明な点が多く残されていた．最近の報告で，DEC-205がTLR9の合成リガンドであるK型のCpG ODNの細胞表面受容体であることが報告された[28)]．TLR9はエンドソーム内に局在しており，長らくどのようにしてCpG ODNがエンドソームに取り込まれ，TLR9によって認識されるのか明らかとなっていなかった．K型のCpG ODNは骨格がホスホロチオエート化されており，DEC-205は直接結合すること，そしてDEC-205欠損細胞ではCpG ODNによる自然免疫応答の減弱が確認された(図4)．この結果は，今後CpG ODNを臨床で用いるためには重要な発見であり，DEC-205発現細胞にCpG ODNを効率的にターゲットすることで，新たな核酸医薬の開発にもつながることが期待される．

Ly49Qはimmunoreceptor tyrosine-based inhibitory motif(ITIM)を有しており，ナチュラルキラー(NK)受容体ファミリーに属しているCLRsである．しかしながら，Ly49QはNK細胞に発現しておらず，pDCで強く発現していることが確認されている．Ly49Qの内因性のリガンドとして，自己のMHCクラスI分子が報告されていたが，pDCとMHCクラスIとの結合と自然免疫応答に関しては不明な点が残されていた[29)]．最近の報告で，Ly49QによるMHCクラスIの認識が，pDCからのIFN-α産生に必須であることが明らかとされ

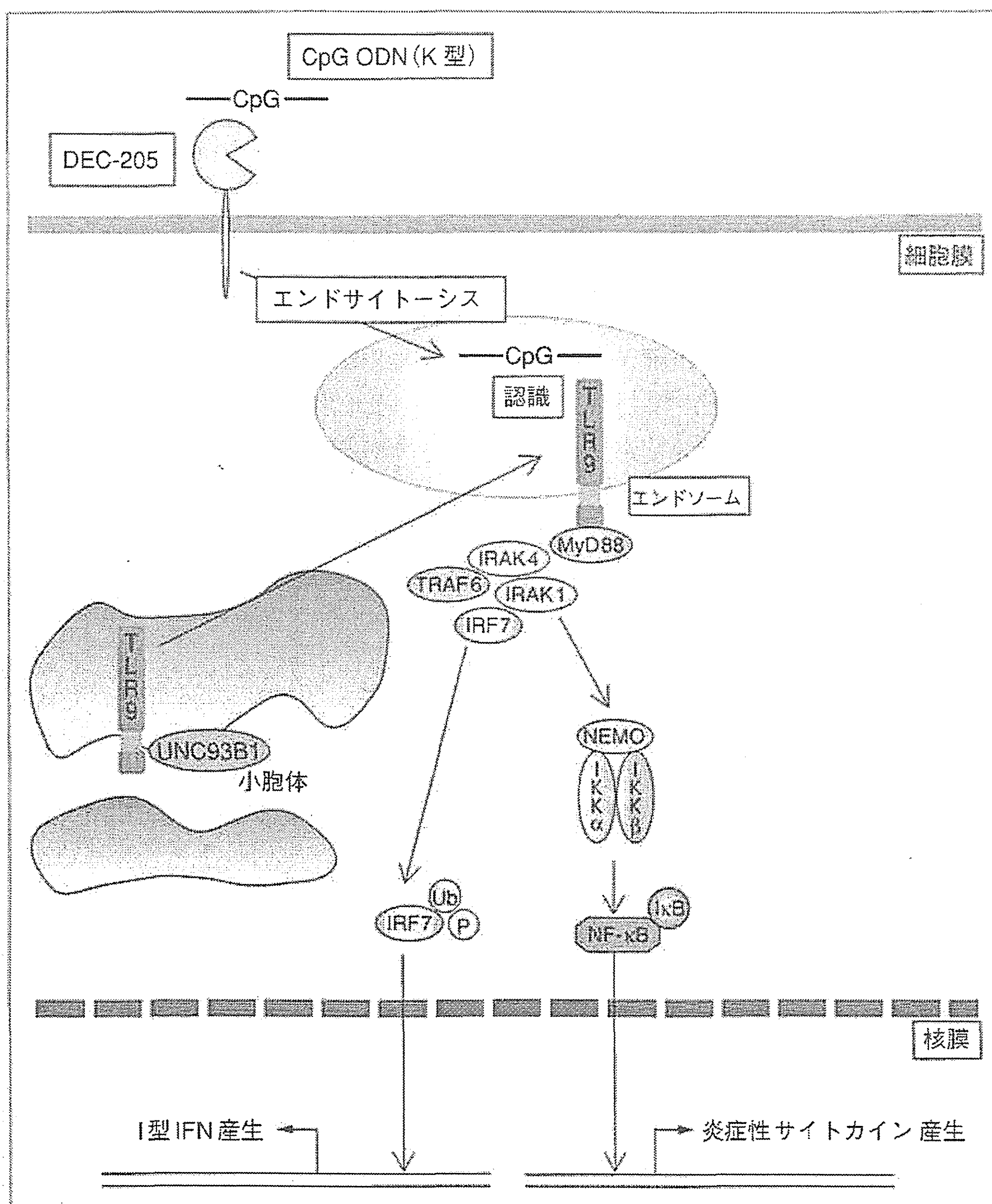

図4　CLRsとTLRsによる共同作用

た[30]．実際に，CpG ODN刺激によるIFN-αの産生は，Ly49Qまたは，MHCクラスⅠの抗体を用いてお互いの結合を阻害することで抑制された．また，Ly49Qを欠損しているpDCでは，CpG ODN刺激による著しいIFN-α産生の抑制が確認されており，同時にインフルエンザウイルスやサイトメガロウイルス感染後のIFN産生の抑制も確認されている．これらの結果は，Ly49QとMHCクラスⅠとの結合が，pDCによるサイトカイン産生をポジティブに制御していることを示唆している．また，サイトメガロウイルス感染などによって発現が低下するMHCクラスⅠが，pDCによる抗ウイルス応答や，CTLの活性化を抑制することにも関係していると考えられる．

おわりに

さまざまな自然免疫受容体で病原体を認識することは，宿主側にいくつかのアドバンテージを与えることができる．①いくつかの受容体で認識することで，病原体による免疫のエスケープ機構に対抗することができる．たった一つの自然免疫受容体による認識は回避できても，いくつもの自然免疫受容体から回避するのは，病原体側からしても容易ではない，②宿主の遺伝

的多様性，③より強い炎症性反応を誘導することができる，④宿主にとって適切な炎症反応を誘導することが考えられる．このように，宿主はいくつもの自然免疫受容体を備えることで，さまざまな場面に対して，より正確に，より強力に炎症反応を誘導し，最適な獲得免疫応答を誘導できていると考えられる．これまでの研究は，一つの自然免疫受容体に対して，いくつかの病原体を解析することが主流だったが，今後は特定の病原体に対して，どのようにそれぞれの自然免疫受容体が関与し，病原体から宿主を守っているのか明らかにしていく必要がある．実際に，ワクチン開発を考えてみても，どの自然免疫受容体を活性化することが必須であるのか，特定の病原体に対して理解が深まっていれば，より効果的なワクチンの開発が進んでいく．今後の研究によって，そのようなワクチンの開発が達成されることを期待したい．

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Isolation and Characterization of Highly Replicable Hepatitis C Virus Genotype 1a Strain HCV-RMT

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Abstract

Multiple genotype 1a clones have been reported, including the very first hepatitis C virus (HCV) clone called H77. The replication ability of some of these clones has been confirmed *in vitro* and *in vivo*, although this ability is somehow compromised. We now report a newly isolated genotype 1a clone, designated HCV-RMT, which has the ability to replicate efficiently in patients, chimeric mice with humanized liver, and cultured cells. An authentic subgenomic replicon cell line was established from the HCV-RMT sequence with spontaneous introduction of three adaptive mutations, which were later confirmed to be responsible for efficient replication in HuH-7 cells as both subgenomic replicon RNA and viral genome RNA. Following transfection, the HCV-RMT RNA genome with three adaptive mutations was maintained for more than 2 months in HuH-7 cells. One clone selected from the transfected cells had a high copy number, and its supernatant could infect naïve HuH-7 cells. Direct injection of wild-type HCV-RMT RNA into the liver of chimeric mice with humanized liver resulted in vigorous replication, similar to inoculation with the parental patient's serum. A study of virus replication using HCV-RMT derivatives with various combinations of adaptive mutations revealed a clear inversely proportional relationship between *in vitro* and *in vivo* replication abilities. Thus, we suggest that HCV-RMT and its derivatives are important tools for HCV genotype 1a research and for determining the mechanism of HCV replication *in vitro* and *in vivo*.

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Introduction

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus that belongs to the Flaviviridae family [1]. HCV infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. With over 170 million people currently infected worldwide [2], HCV represents a growing public health burden despite the launch of new antiviral medications that directly inhibit virus replication [3,4].

Since HCV was first identified in 1989 as the major cause of non-A and non-B hepatitis [5], great progress has been made in understanding the life cycle of HCV. The first propagation system for this disease agent was an *in vivo* chimpanzee model [6,7,8]. Although that system is still occasionally used as a pivotal animal model for some drugs, chimeric mice with humanized liver that is generated by transplanting human hepatocytes [9,10] are more popular now because of the low cost and the absence of ethical concerns associated with the use of chimpanzees. For *in vitro* research, establishment of an HCV replicon system [11,12] was an important achievement that allowed research into the function of individual non-structural viral proteins. However, the entire viral life cycle remains enigmatic because no structural proteins are needed in this system. Some reports have been published about full-length replicons with structural proteins in addition to non-structural proteins, although little [13] or no [14,15] secretion of infectious virions was observed, which may have been partly due to adaptive mutations. Another breakthrough was made with the discovery of a genotype 2a Japan fulminant hepatitis (JFH)-1 strain that soon became well known for its vigorous replication as a replicon with no adaptive mutations [16]. JFH-1 can also infect and propagate in cultured cells as a virus, especially in HuH-7 cells or their derivatives [17–19]. After the discovery of JFH-1, two methods were available for the investigation of how viral proteins other than those of HCV genotype 2a function during their entire life cycle. The first method was only for structural proteins and involved making a hybrid of the structural region of the clone of interest and the non-structural regions of JFH-1 for efficient replication [20–22]. The other method utilized the entire viral genome sequence of genotype 1 and made them infectious to HuH-7 derivative cells by introducing known adaptive mutations [23,24] or enhancing replication with a casein kinase inhibitor [25]; however, their replication abilities were somehow compromised. In this study, we report the isolation of a new genotype 1a strain from a patient's serum sample that was highly infectious to human hepatocyte-transplanted chimeric mice, as the viral titer in the blood of the mice was higher than 10^8 copies/ml. We

evaluated its replication abilities in four replication systems: subgenomic replicon, virus, *in vitro* infection, and *in vivo* infection. The new HCV clone, which was designated HCV-RMT (GenBank accession number, AB520610), was different from other genotype 1a clones because it did not require any artificially introduced adaptive mutations for the establishment of replicon cells. With these features, our newly cloned HCV-RMT may be a useful tool for investigating the entire life cycle of genotype 1 HCV.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with both the *Guidelines for Animal Experimentation* of the Japanese Association for Laboratory Animal Science and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All protocols were approved by the ethics committee of Tokyo Metropolitan Institute of Medical Science.

Cloning and Sequencing

Acute-phase serum from an HCV genotype 1a-infected patient, HCG9 (purchased from International Reagents Corp., Kobe, Japan; discontinued), was supplemented with 0.1 μg/μl yeast tRNA, and total RNA was extracted using ISOGEN-LS (Nippon Gene, Tokyo, Japan) according to the manufacturer's information. Purified RNA (1 μg) was reverse transcribed using LongRange Reverse transcriptase (QIAGEN, Valencia, CA, USA) and a 21-mer oligonucleotide (antisense sequence 9549-9569 of HCV-H77: GenBank accession number AF011751) as the primer. The first PCR amplification was carried out with the generated cDNA and Phusion DNA polymerase (Finnzymes, Vantaa, Finland) using sense primers corresponding to nucleotides 9-28, 2952-2972, and 5963-5979 (numbers correspond to the HCV-H77 sequence) and antisense primers corresponding to nucleotides 4038-4054, 7042-7057, and 9549-9569. The second nested PCR amplification was carried out with these three products using sense primers corresponding to nucleotides 23-43, 2967-2987, and 5981-6000 and antisense primers corresponding to nucleotides 4018-4033, 7016-7035, and 9534-9554. For the cloning of terminals, total RNA was purified from non-supplemented HCG9 serum. The 5′ terminus was amplified with a 5′ RACE system kit (Invitrogen, Carlsbad, CA, USA) using one-fourth of the purified total RNA from 100 μl serum and antisense primers corresponding to nucleotides 255–273 for the first PCR and 241–261 for the second nested PCR. For the 3′ terminus, the poly(A) tail was added to the 3′ terminus of the same amount of RNA with poly(A) polymerase (Takara Bio Inc., Shiga, Japan). Reverse transcription and PCR amplification of this region were carried out using oligo-d(T) as the reverse primer for both reactions and primers corresponding to nucleotides 9385-9408 for PCR.

All fragments were subcloned using a TOPO cloning kit (Invitrogen), and sequences yielding 10 or more clones per fragment were determined with the Big Dye Terminator mix and ABIprism3100 (Applied Biosystems, Foster City, CA, USA). The consensus sequence was determined by accepting the most frequent nucleotide at each position.

Construction and RNA transcription

To generate full-length viral RNA, the HCV-RMT sequence, which has an endogenous *Xba*I site, was mutated to a silent mutation (T3941C) using a QuikChangeII kit (Stratagene, La Jolla, CA, USA) and cloned into the *Hind*III site of pBR322 with an additional T7 promoter at the beginning and an *Xba*I site at the end. Replicon construction of HCV-RMT was performed by replacing nucleotides 390-3419 of HCV-RMT with the neomycin resistance gene, encephalomyocarditis virus internal ribosome entry site (EMCV-IRES), and an additional start codon at the beginning of the NS3 region. For RNA generation, plasmids were digested with *Xba*I and used as a template for RNA transcription using a RiboMax kit (Promega, Madison, WI, USA).

Cells and Electroporation

HuH-7 cells were cultured in DMEM-GlutaMax-I (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Replicon cells were maintained in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Cells were passaged three times a week at a split of four times. Electroporation of replicon RNA and G418 selection were performed as previously described [11,12]. The cured replicon cell clone (HuH7-K4) was established as previously described [13]. Briefly, authentic subgenomic replicon cells were treated with 1000 IU interferon-α (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) for 2 months and cloned using the limiting dilution method.

Quantification of HCV RNA

Total RNA was purified from 1 μl chimeric mouse serum using SepaGene RV-R (Sanko Junyaku, Tokyo, Japan), and total RNA was prepared from cells or liver tissues using the acid guanidium thiocyanate-phenol-chloroform extraction method. Quantification of HCV RNA copy number with real-time RT-PCR was performed using an ABI 7700 system (Applied Biosystems) as described previously [26].

Western blot analysis and immunofluorescence analysis

Western blot analysis was carried out according to the conventional semi-dry blot method. Cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4 containing 1% sodium dodecyl sulfate, 0.5% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 1 mM dithiothreitol). Protein (10 μg) from each sample was separated with SDS-PAGE through a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore, Billerica, MA, USA). HCV NS3 protein was detected with 5 μg/ml anti-NS3 polyclonal antibody (R212) as described previously [27]. HCV NS5B protein and β-actin were detected with 0.5 μg/ml anti-NS5B polyclonal antibody (ab35586; Abcam, Cambridge, UK) and 0.2 μg/ml anti-β-actin monoclonal antibody (AC-15; Sigma-Aldrich, St. Louis, MO, USA), respectively.

For immunofluorescence analysis, cells were washed twice with PBS(−) and fixed with 100% methanol (chilled at −80°C) at −20°C for 20 min. Fixed cells were treated with PBS(−) supplemented with 1% BSA and 2.5 mM EDTA overnight at 4°C. Blocking and antibody treatments were also carried out in the same buffer. Stained cells were viewed with a laser scanning confocal microscope LSM510 (Carl Zeiss, Oberkochen, Germany). HCV core proteins were detected with 5 μg/ml α-HCV core monoclonal antibody (31-2) prepared in our laboratory [28].

In vitro infection and α-CD81 blocking

HuH7-K4 cells were seeded at 6×10^4 cells/well onto a φ10-mm coverglass in a 48-well plate 24 h before inoculation with 240 μl culture medium. At 72 h post-inoculation, cells were fixed with 100% methanol (chilled to −80°C) for 20 min at −20°C. HCV core proteins were detected with 5 μg/ml α-HCV core antibody 31-2. Fluorescent-positive foci were counted under fluorescence microscopy, and the focus-forming units (ffu) per milliliter of supernatant were calculated.

For α-CD81 blocking, HuH7-K4 cells were pre-treated with a serial dilution of α-CD81 antibody (JS81, BD Pharmingen, San Diego, CA, USA) or normal mouse IgG_1 (BD Pharmingen) as an isotype control for 1 h before inoculation.

Drug treatment

#11 cells (5,000 cells/well), which were established using the single cell cloning of HCV-RMTtri-electroporated cells, were seeded in 96-well tissue culture plates and cultivated overnight. Serial dilutions of cyclosporin A (Fluka Chemie, Buchs, Switzerland) or interferon-α (Mochida Pharmaceutical Co., Ltd.) were added. After incubation for 72 h, total RNA was extracted from cells, and HCV-RNA was quantified as described above. The experiments were carried out in triplicate.

In vivo infection

Chimeric mice with humanized liver (PhoenixBio, Hiroshima, Japan) were infected with 10 µl patient serum HCG9 by intravenous injection. For analysis of infectivity of the HCV genome clone, mice were directly injected with 30 µg of the generated RNAs into five to six sites in the liver during abdominal surgery. Blood samples were collected once a week and used for quantification of HCV copy number.

Results

Cloning of a new HCV genotype 1a strain from the serum of an HCG9-infected mouse

We first infected chimeric mice with humanized liver with patient serum HCG9. HCV in HCG9 serum was classified as genotype 1a with RT-PCR genotyping and showed a relatively high replication ability in the patient and a comparable or better replication ability in the chimeric mice (Figure 1A). In one infected mouse, the HCV copy number in blood reached 1×10^9/ml (data not shown). Using two mice with blood titers of 1×10^8 and 1×10^9 copies/ml, we cloned HCV sequences with the standard PCR amplification method using HCV-H77 as a source of primer sequences. Except for some length variations in the poly-pyrimidine tract region, we found no differences in HCV sequences from mouse blood with titers of 1×10^8 and 1×10^9 copies/ml when considering major consensus nucleotides at all sites (GenBank accession number AB520610). The HCV sequences were identical to the HCV sequence cloned from HCG9 serum itself (data not shown). We designated this sequence as HCV-RMT. Its homology to the HCV-H77 strain was 92.8% for nucleotides and 95.1% for amino acids. The *in vivo* replication ability was confirmed with direct injection of the generated HCV-RMT RNA genome into livers of the chimeric mice. Blood titers were comparable to infection with parental HCG9 serum (Figure 1B). JFH-1 infection resulted in a 2-log lower blood titer than HCV-RMT when the same procedure was used.

Establishment of subgenomic replicon cells with the HCV-RMT strain

Next, we generated an authentic subgenomic replicon RNA construct using the HCV-RMT sequence and used it to establish replicon cells. Only two colonies appeared after electroporation with 30 µg of the HCV-RMT replicon RNA and G418 selection. One of these colonies had a reasonable HCV subgenome copy number, and thus, we propagated it and determined the sequence of the subgenome. The determined consensus sequence of the subgenome had three mutations from the wild type: two were located in the NS3 region (E1056V and E1202G), and one was in the NS5A region (A2199T) (Figure 1C). We introduced these mutations into the HCV-RMT replicon sequence as a single mutation or combination of mutations and identified the mutations that were responsible for colony formation (Figure 1D). The most influential single mutation was E1202G in the NS3 region, although a combination of all three mutations (designated RMTtri) resulted in the best replication ability. Interestingly, western blot analysis and HCV genome quantification revealed that the amount of HCV viral components in cells was independent of the colony-forming ability and seemed to be negatively affected by the most beneficial adaptive mutation (E1202G) (Figure 1E).

The HCV-RMT RNA genome with adaptive mutations was maintained in HuH-7 cells

Next, we assessed the *in vitro* replication abilities of HCV-RMT derivatives as a viral genome rather than a replicon. We introduced adaptive mutation(s) into the HCV-RMT sequence (Figure 2A) and electroporated the *in vitro*-generated RNAs into Huh-7.5.1 and HuH7-K4 cells. Electroporated cells were passaged every 2 to 4 days depending on their confluency, and sampling of cells for quantification of the HCV RNA genome was carried out at each passage. The amounts of HCV-RMTtri and JFH-1 were maintained at $\geq 1\times10^5$ copies/µg total RNA, in contrast to wild-type HCV-RMT, which was eliminated rapidly (Figure 2B). Additionally, different cell preferences were observed with the two strains of HCV: JFH-1 replicated well in Huh-7.5.1 cells compared to HCV-RMTtri, but the opposite was seen in HuH7-K4 cells. These tendencies were observed repeatedly (Figure S1). Different replication abilities were also observed among derivatives of the HCV-RMT strain and corresponded to the colony-forming ability of the replicon constructs (Figure 2C). Immunostaining of HCV core proteins revealed that many cells (19.2%) were stained in HCV-RMTtri RNA electroporated cells compared to small number cells (0.98%) were stained in HCV-RMT with E1202G mutated RNA electroporated cells (Figure 2D).

The supernatant of HCV-RMTtri-replicating cells was infectious to naïve HuH7-K4 cells

To assess the infectivity of HCV-RMTtri, we used the limiting dilution method to establish clone number 11 (#11) cells in which HCV-RMTtri was highly replicating. The percent of cells expressing the HCV core protein in #11 cells was 75.3±5.0% as seen with immunostaining, whereas the percent of parental cells expressing the HCV core protein was 6.3±2.2% (Figure 3A; the value was calculated as an average of ten observed areas). The cells maintained 1×10^8 copies/µg total RNA of the HCV-RMTtri RNA genome. We collected the supernatants from #11 cells 2 months after cloning and HuH7-K4 cells carrying JFH-1 2 months after establishment. To evaluate infectivity, we added these supernatants to the medium of naïve HuH7-K4 cells. Cells were stained with anti-HCV core protein antibody 3 days later, and we observed core protein-positive cell foci per 0.78 cm^2 in at least triplicate wells (Figure 3B). The calculated ffu of the supernatant was 160 ffu/ml, which was similar to that of H77 with artificially introduced adaptive mutations. This infection was inhibited by anti-CD81 antibody in a similar concentration-dependent manner as *in vitro* infection of JFH-1 (Figure 3C). #11 cells were also useful for evaluating anti-HCV agents such as cyclosporin A and interferon-α (Figure 3D) when 5,000 cells/well (96 well plate) of #11 cells were treated with inhibitors for 72 h beginning 1 day after passaging.

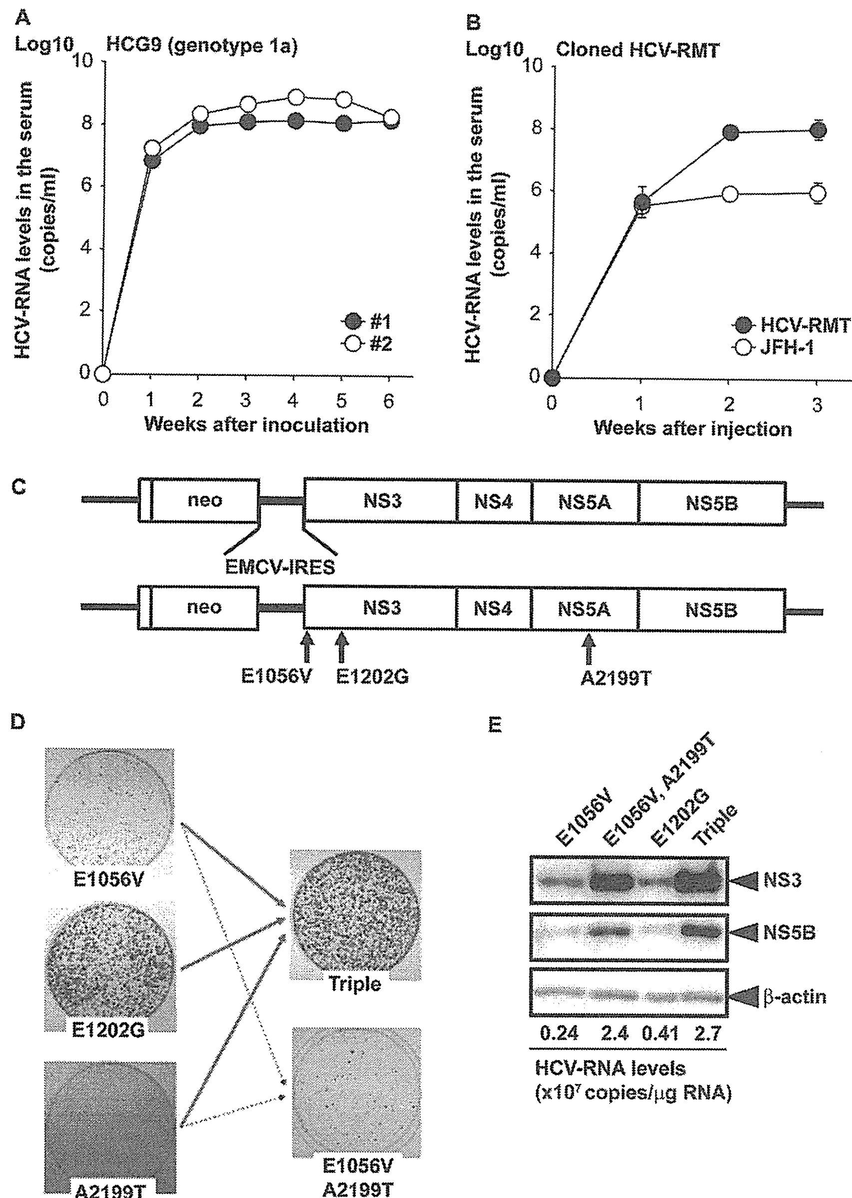

Figure 1. Basic characteristics of the HCV-RMT clone. Change in HCV copy number in chimeric mice. (A) Two mice were intravenously infected with 10 μl patient serum HCG9. (B) Three mice per group were directly injected with 30 μg HCV RNAs of the HCV-RMT strain or the JFH-1 strain into the liver. Data are indicated as the mean ± S.D. (C) Schematic representation of construction of the replicon and the sites of adaptive mutations. (D) Colony formation assay of replicon clones with adaptive mutations. Each RNA (1 μg) was electroporated into HuH7-K4 cells. (E) Western blot analysis of replicon cells. Each culture of replicon RNA-electroporated cells was maintained and passaged with G418 selection for 2 weeks. Cell lysates (10 μg) were loaded onto an SDS-PAGE gel.
doi:10.1371/journal.pone.0082527.g001

In vivo replication abilities of HCV-RMT derivatives were inversely proportional to their *in vitro* abilities

We assessed the *in vivo* replication abilities of HCV-RMT derivatives carrying combinations of the three adaptive mutations using chimeric mice with humanized liver. *In vitro*-generated HCV genomic RNAs were injected directly into the livers of the chimeric mice during abdominal surgery. Mice were monitored for amounts of genomic RNA in the blood once a week for 6 weeks, and virus titers in the livers were quantified after sacrifice of the mice. As shown in Figure 4A, in contrast to the vigorous *in vitro* replication ability, the clone that was most active *in vitro*, HCV-RMTtri, showed no evidence of replication *in vivo*, whereas the wild type showed replication that was comparable to the parental virus in the patient's serum, HCG9. In addition, the double mutant (E1056V, A2199T), which showed little replication *in vitro*, showed a similar replication ability as the wild-type clone. The most positively influential adaptive mutation (E1202G) seemed to

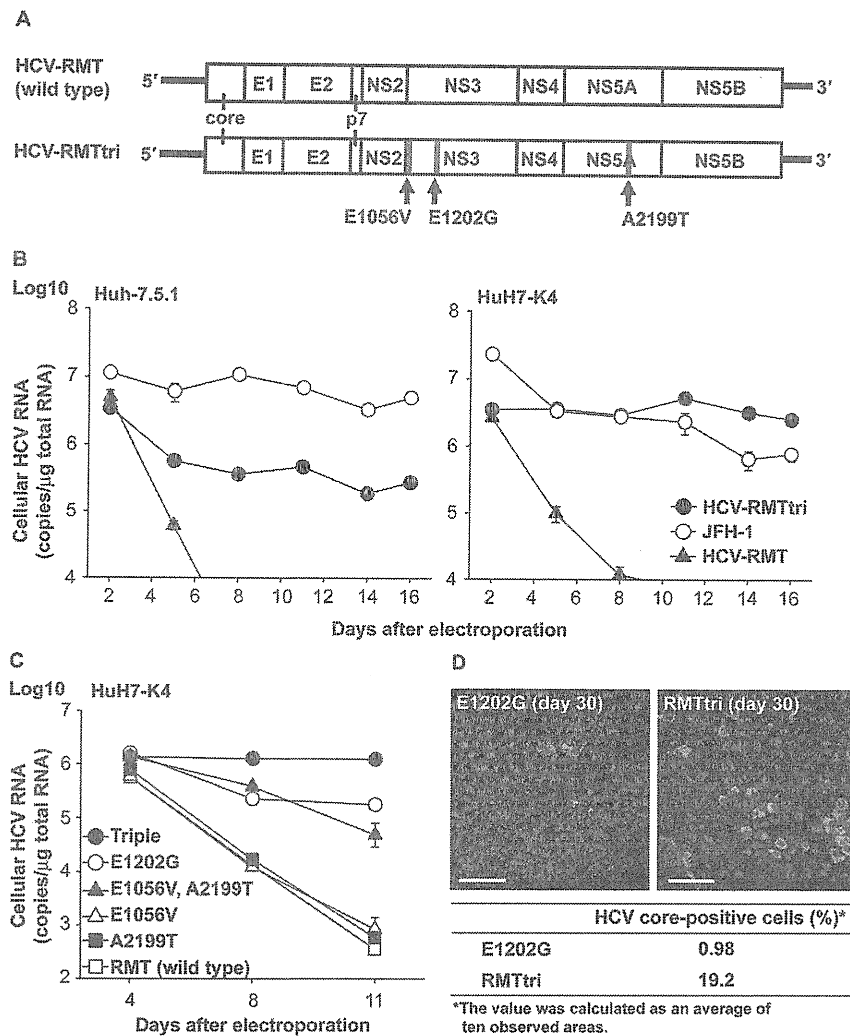

Figure 2. *In vitro* replication ability of HCV-RMT derivative genomes. (A) Schematic representation of construction of the HCV genome and the sites of adaptive mutations (red bars). (B) Electroporation of the generated HCV-RNA genomes of wild-type HCV-RMT (closed triangles), HCV-RMT with triple mutations (HCV-RMTtri; closed circles), and the JFH-1 strain (open circles) into Huh-7.5.1 or HuH7-K4 cells. The experiments were carried out in duplicate. (C) Comparison of the *in vitro* replication ability of each HCV-RMT derivative in HuH7-K4 cells. The experiments were carried out in duplicate. Wild type: open squares, E1202G: open circles, E1056V: open triangles, A2199T: closed squares, E1056V and A2199T: closed triangles, triple mutations: closed circles. (D) Immunostaining for the HCV core protein in HCV-RNA-electroporated cells. Scale bar = 100 μm. The percent of HCV core protein-positive cells (%) was calculated as an average of ten observed areas.
doi:10.1371/journal.pone.0082527.g002

hamper its *in vivo* replication ability. Quantification of HCV genomic RNA in liver (Figure 4B, C) showed a conserved serum/liver ratio among HCV-RMT derivatives. Thus, the blood titers directly reflected the titers in liver, although the ratio was considerably different than that of JFH-1. Table 1 shows the replication abilities of derivatives and JFH-1 both *in vitro* (HuH7-K4 cells) and *in vivo* (chimeric mice), clearly showing the inversely proportional relationship between them, including the replication ability of JFH-1, which corresponds to data in a previous report [29].

Discussion

In this report, we investigated many types of HCV replication systems using our newly cloned HCV-RMT.

The first type is replication in cultured cells as an authentic replicon construction. This system only depends on the ability to replicate in cells. HCV-RMT was the first genotype 1a clone that could be established in authentic replicon cells without artificially introduced adaptive mutations that are required by H77 [30,31], although the three spontaneously occurring mutations (E1056V, E1202G, and A2199T) are not novel [11,31]. Among the mutants with single mutations or a combination of these three adaptive mutations, the amounts of HCV genome and viral proteins did not reflect the colony-forming abilities (Figure 1D, E). The A2199T mutation, which least affects the colony-forming ability (no stable replicon cell line was established with this single mutation). However, combination of mutations including A2199T, triple (E1056V, E1202G and A2199T) and double (E1056V and

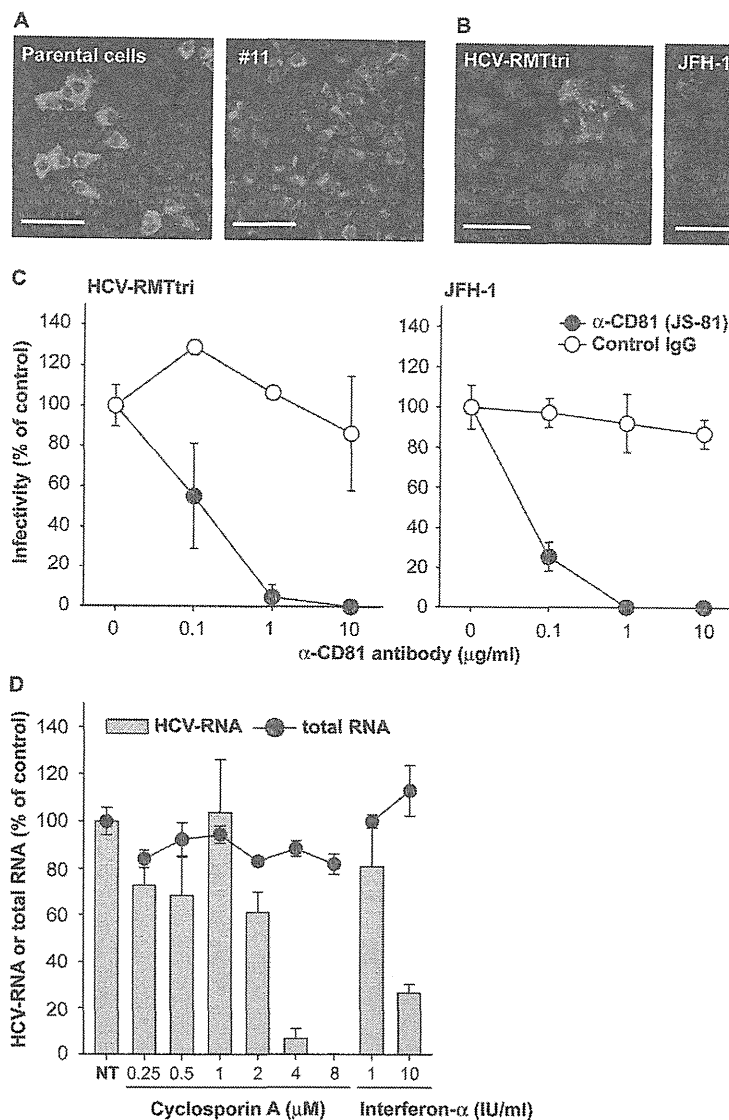

Figure 3. Establishment of HCV-RMTtri highly replicating #11 cell and infectivity of its supernatant on naïve HuH7-K4 cells. (A) Immunostaining for the HCV core protein in HCV-RMTtri-electroporated parental cells and the cell clone (#11) obtained by limiting dilution cloning. Scale bar = 100 μm. (B) Immunostaining for the HCV core protein in naïve HuH7-K4 cells infected with supernatants of HCV-RMTtri- or JFH-1-replicating cells. Scale bar = 50 μm. (C) Infection with the HCV-RMTtri supernatant was inhibited with anti-CD81 antibody in a similar manner as JFH-1. Control IgG (normal mouse IgG_1): open circles, anti-CD81 mAb (JS-81): closed circles. Data are indicated as the mean ± S.D. (D) Replication of HCV-RMTtri in HuH7-K4 cells was inhibited by HCV replication inhibitors such as cyclosporin A and interferon-α. Drugs were added to #11 cells in 96-well plates 1 day after passaging, and cells were harvested after 72 h of treatment. NT: no treatment.
doi:10.1371/journal.pone.0082527.g003

A2199T), allowed HCV subgenomic replicon cells to produce high amounts of HCV proteins. This observation illustrates the complex nature of HCV subgenomic replicon-establishing factors, especially NS5A-related factors [32,33]. This hypothesis requires further investigation.

The second type is replication of the virus itself in cultured cells. This system also only depends on the ability to replicate in cells as long as the presence of structural protein regions does not cause any differences. Electroporation of HCV genomes resulted in constant replication when the combination of active derivatives of HCV-RMT and HuH7-K4 cells was used; replication lasted for more than 2 months (data not shown). The order of the replication ability of mutants in cultured cells as a virus appeared to be nearly consistent with the colony-forming ability of replicons of each sequence, although some constructs with "weak" adaptive mutation(s) showed no difference from the wild type (Figure 2C). Thus, these two types of replication may be basically the same despite the different constructs. HCV-RMT derivatives replicated better than JFH-1 in HuH7-K4 cells. In contrast, replication was much less efficient in Huh-7.5.1 cells (Figure 2B), which are well known to support replication of JFH-1 [34]. These materials

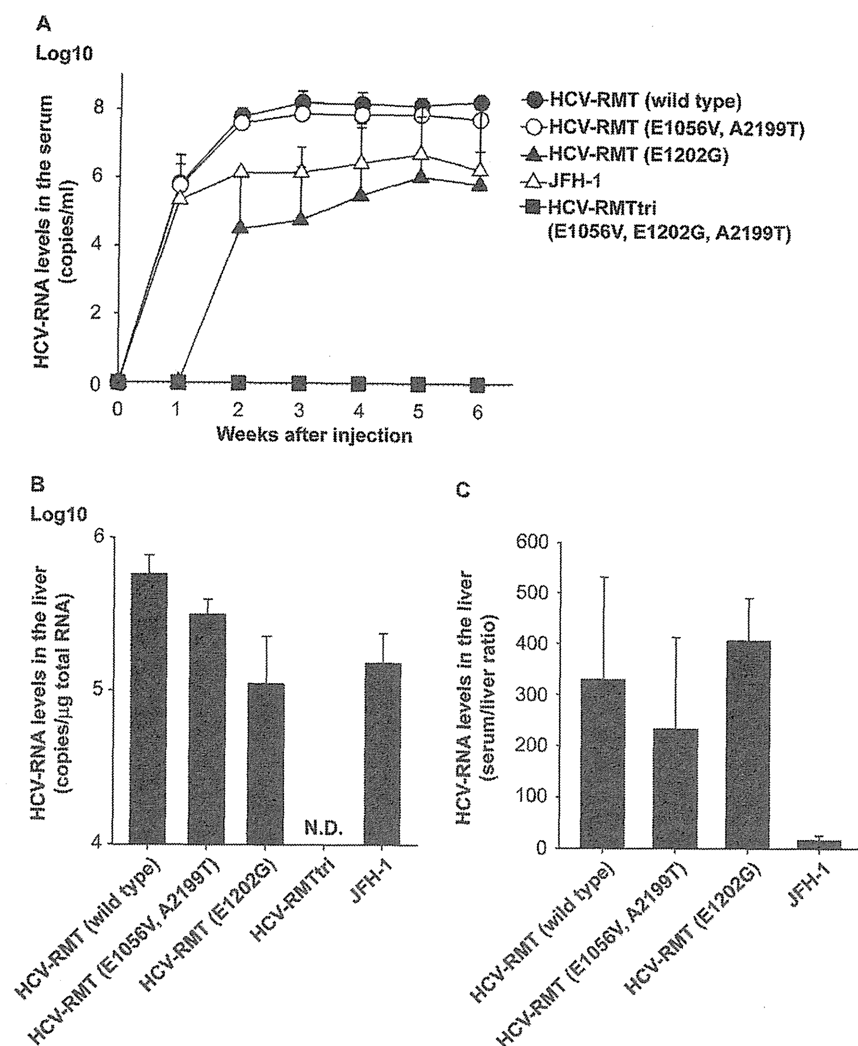

Figure 4. ***In vivo*** **replication ability of HCV-RMT derivatives.** (A) Change in HCV copy numbers in the serum of chimeric mice in which the HCV genome was directly injected into the livers. HCV-RMT (wild type): closed circles, HCV-RMT (E1056V and A2199T): open circles, HCV-RMT (E1202G): closed triangles, HCV-RMTtri: closed squares, JFH-1: open triangles. Data are indicated as the mean ± S.D. (B) HCV copy number in the livers. N.D.: not detected. (C) Serum/liver ratio of HCV copy number.
doi:10.1371/journal.pone.0082527.g004

Table 1. Relationship between the *in vitro* and *in vivo* replication ability of HCV-RMT derivatives.

Clones	*in vitro*	*in vivo*
HCV-RMT (wild type)	-	+++
HCV-RMT (E1056V, A2199T)	-	+++
HCV-RMT (E1202G)	+	+
HCV-RMTtri	+++	-
JFH-1	++	+

For the *in vitro* column, +++: maximum replication ability, ++: approximately 1 log lower than the maximum, +: approximately 2 logs lower than the maximum, -: no difference compared to the wild-type strain. For the *in vivo* column, +++: maximum replication ability, ++: approximately 1 log lower than the maximum, +: approximately 2 logs lower than the maximum, -: no replication.
doi:10.1371/journal.pone.0082527.t001

appear to be good tools for investigating the mechanism of HCV replication in cultured cells.

The third type is *in vitro* infection using established HCV-infected cultured cells as the source of inoculum. Because this system has more steps than the first two, the outcome is more difficult to understand. Infection systems using strains other than JFH-1 seem to be rare because the magnitude of their replication is somehow compromised [23,25,35], in contrast to several studies examining the JFH-1 strain or its chimeric constructs with other genotypes [17–22]. For observation of the infection process, selection of efficiently replicating cell clones from HCV-RMT RNA-electroporated cells was required. That clone, designated HuH7-K4-#11 cells, had approximately 1×10^{8} copies/µg total RNA of the HCV-RMT genome, and more than 80% of cells were core protein positive (Figure 3A). We were able to infect naïve HuH7-K4 cells with its supernatant (Figure 3), and the infectivity reached approximately 160 ffu/ml, which was compa-

rable to that of the artificially mutated H77 strain. Thus, our HCV-RMT strain was unique among all genotype 1a clones. We could only detect infectivity using HCV-RMTtri, likely because the abundance of HCV-positive cells was high compared to other mutants (data not shown).

Many reports investigating the cellular and/or viral factors required for *in vitro* infection of HCV have been published [36–45]. Almost all of these reports used a combination of JFH-1 and Huh-7.5 or Huh-7.5.1 cells. Our system using genotype 1a HCV-RMT and HuH7-K4 cells could complement these studies, considering the fact that the replication abilities of HCV-RMT and JFH-1 were quite different in the two derivatives of HuH-7 cells (Figure 2).

Among the host factors reported to influence the virus life cycle *in vitro* and *in vivo*, CD81 was the first reported receptor to be involved in the *in vitro* infection process [22,36-39], and CD81 was also necessary in our HCV-RMT infection system because infection was blocked by anti-CD81 mAb (Figure 3).

Pietschmann et al. reported that the production of virus particles is impaired by replication-enhancing mutations [23]. Because we could not detect any infectivity in any supernatants from HCV-RMT derivative-electroporated cells at early times, we could not determine whether this idea applies to these derivatives. Our observation of *in vitro* infectivity only with HCV-RMTtri may be due to the balance of replication ability and budding ability. Our system may be sufficiently efficient to quantify the infectivity titer.

Other cellular factors such as lipid droplets that interact with core proteins [40–42] and apolipoproteins [43–45] have been reported previously, although their contribution to our new infection system requires further studies.

The last type of HCV replication system we investigated was *in vivo* infection. The chimpanzee model was the first animal model established for HCV infection and was frequently used in important studies despite its high cost and ethical problems. Studies using chimpanzees have revealed that *in vitro*-adapted HCV mutants require back mutation(s) at specific site(s) for efficient replication *in vivo* [46]. In our studies using chimeric mice with humanized liver, we also did not observe amplification of HCV-RMTtri, which was the most active *in vitro* mutant. In addition, we observed a clear inversely proportional relationship between the *in vitro* and *in vivo* replication abilities of each mutant (Table 1), suggesting that the same factor(s) may work in both *in vitro* replication enhancement and *in vivo* replication inhibition. Although we have not confirmed whether back mutations or other new complimentary mutations were present, the characteristics of these three mutations were clarified by analysis of these four types of replication: replicon, virus, *in vitro* infection, and *in vivo* infection. E1202G, one of the two NS3-adaptive mutations, was the most important mutation for *in vitro* replication, but it also severely hampered *in vivo* amplification. This mutation appears to impact the colony-forming ability comparable to the triple mutation, although virus replication was relatively lower than with the triple mutation. E1056V, another NS3 mutation, had a mild impact on the colony-forming ability, but it did not hamper the efficient replication of wild-type RMT *in vivo* in combination with A2199T, which seemed to have little influence on HCV replication alone except for increasing the amounts of virus genome and viral proteins in replicon cells. These two "weak" adaptive mutations provide the E1202G single mutant the ability to efficiently replicate *in vitro*. These effects may be dependent on the colony-forming ability of E1056V, the genome- and protein-increasing ability of A2199T, or both. At the same time, the weak *in vivo* replication ability of the E1202G single mutant, the replication of which was detected in only two of three mice injected and which showed a relatively low titer, was destroyed by addition of these two mutations, although the combination of these mutants had little effect on the replication ability of the wild type. These results suggest that the putative mechanism that renders *in vitro*-active clones deficient *in vivo* is not caused by a single factor such as the phosphorylation status of the NS5A protein, but a balance of many factors controlling mechanisms that are directly related to HCV replication both *in vitro* and *in vivo*.

We evaluated the amounts of HCV genome both in blood and liver, and the blood:liver ratios of replicable mutants varied little. This observation seems to be inconsistent with the hypothesis that the *in vivo* ability of *in vitro* active mutants is compromised because of adaptive mutations that make the HCV genome more replicable but impair its virion-producing ability. Whether this occurs in certain conditions or is universal must be elucidated.

Recently, Li et al. reported the efficient replication and infection of Huh-7.5 cells of a genotype 1a clone named TN with artificially introduced adaptive mutations [24]. Similar reports have been published regarding an infectious genotype 1 HCV genome in Huh-7.5 cells or their derivatives by introducing adaptive mutations or using replication enhancing reagent [23,25]. Although these appear to be more infectious than our HCV-RMT strain, we believe that our system is valuable because of the cells we used. HuH7-K4 cells are not a derivative of Huh-7.5 cells and are apparently distinct from them in terms of the ability to support HCV replication (Figure 2).

Our newly cloned HCV-RMT strain is unique because of its vigorous replication ability in chimeric mice, compared to the first HCV strain, H77, or the JFH-1 strain that is well known for its efficient replication *in vitro*. We believe that the different levels of *in vitro* replication abilities of these HCV-RMT mutants with an inversely proportional relationship to *in vivo* replication are valuable tools for investigating the factors required for HCV replication both *in vitro* and *in vivo*.

Supporting Information

Figure S1 *In vitro* replication ability of HCV-RMT derivative genomes. Electroporation of the generated HCV-RNA genomes of wild-type HCV-RMT (closed triangles) and HCV-RMT with triple mutations (HCV-RMTtri; closed circles) into HuH7-K4 cells. The experiments were carried out in duplicate.
(TIF)

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Author Contributions

Conceived and designed the experiments: MK. Performed the experiments: MA Y. Tokunaga AT Y. Tobita YH YI CT. Analyzed the data: MA Y. Tokunaga. Wrote the paper: MA.

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BASIC AND TRANSLATIONAL—LIVER

A Serine Palmitoyltransferase Inhibitor Blocks Hepatitis C Virus Replication in Human Hepatocytes

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See Covering the Cover synopsis on page 701.

BACKGROUND & AIMS: Host cell lipid rafts form a scaffold required for replication of hepatitis C virus (HCV). Serine palmitoyltransferases (SPTs) produce sphingolipids, which are essential components of the lipid rafts that associate with HCV nonstructural proteins. Prevention of the de novo synthesis of sphingolipids by an SPT inhibitor disrupts the HCV replication complex and thereby inhibits HCV replication. We investigated the ability of the SPT inhibitor NA808 to prevent HCV replication in cells and mice. **METHODS:** We tested the ability of NA808 to inhibit SPT's enzymatic activity in FLR3-1 replicon cells. We used a replicon system to select for HCV variants that became resistant to NA808 at concentrations 4- to 6-fold the 50% inhibitory concentration, after 14 rounds of cell passage. We assessed the ability of NA808 or telaprevir to inhibit replication of HCV genotypes 1a, 1b, 2a, 3a, and 4a in mice with humanized livers (transplanted with human hepatocytes). NA808 was injected intravenously, with or without pegylated interferon alfa-2a and HCV polymerase and/or protease inhibitors. **RESULTS:** NA808 prevented HCV replication via noncompetitive inhibition of SPT; no resistance mutations developed. NA808 prevented replication of all HCV genotypes tested in mice with humanized livers. Intravenous NA808 significantly reduced viral load in the mice and had synergistic effects with pegylated interferon alfa-2a and HCV polymerase and protease inhibitors. **CONCLUSIONS: The SPT inhibitor NA808 prevents replication of HCV genotypes 1a, 1b, 2a, 3a, and 4a in cultured hepatocytes and in mice with humanized livers. It might be developed for treatment of HCV infection or used in combination with pegylated interferon alfa-2a or HCV polymerase or protease inhibitors.**

Keywords: Direct-Acting Antiviral Agents; DAAs; HCV Lifecycle; Drug.

Hepatitis C virus (HCV) is a major cause of morbidity, affecting approximately 170 million people worldwide.[1] In many cases, HCV results in a persistent infection that evades the host immune response, leading to chronic liver disease, chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[2]

The current therapy for chronic hepatitis C is a combination of weekly injections of pegylated interferon alfa-2a (PEG-IFN) and twice-daily oral doses of ribavirin (RBV). Unfortunately, this combination therapy has limited efficacy and significant side effects.[3,4] Although the HCV NS3/4A protease inhibitors telaprevir and SCH503034 (boceprevir) are approved for the treatment of chronic HCV infection, these compounds must be combined with the current standard of care to be efficacious, and they cannot cure all infected individuals, including IFN-intolerant patients.[5–7] Therefore, antiviral combinations that can achieve superior sustained virologic response without the use of IFN or RBV are needed.

IFN-free combinations of direct-acting antiviral agents (DAAs) have been tested for clinical use as novel anti-HCV therapies.[8–10] Emerging data suggest that DAAs, including NS3/4 serine protease inhibitors, NS5B RNA-dependent RNA polymerase inhibitors, and NS5A inhibitors, when used in combination, can achieve significant antiviral activity, but might select for resistance, which can become a primary cause of treatment failure in clinical studies, especially in difficult-to-treat HCV genotypes.[8,9] Additionally, differences in HCV genotypes can result in reduced antiviral activities of certain DAAs and DAA combinations.[11] Therefore, development of additional antiviral agents with diverse resistance profiles and efficacy against a wide spectrum of HCV genotypes is necessary. Major

*Authors share co-first authorship.

Abbreviations used in this paper: cDNA, complementary DNA; DAA, direct-acting antiviral agent; HCV, hepatitis C virus; IC_{50}, 50% inhibitory concentration; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon alfa-2a; RBV, ribavirin; SPT, serine palmitoyltransferase.

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efforts are underway to identify novel inhibitors and DAA combinations with a high barrier to resistance for the treatment of HCV infection.

We identified a novel class of serine palmitoyltransferase (SPT) inhibitors derived from fungal metabolites that exhibited HCV replication-inhibiting activity.[12] HCV replication occurs on host cell lipid rafts that form a scaffold for the HCV replication complex. Sphingolipids, the downstream products of SPT action, are essential components of lipid rafts associated with HCV non-structural proteins on this microdomain. Prevention of the de novo synthesis of sphingolipids by an SPT inhibitor disrupts the HCV replication complex and thereby inhibits HCV replication. This unique mechanism of host enzyme–targeted viral inhibition was hypothesized to have potential for a high barrier to resistance and for antiviral activity across different HCV genotypes. We identified a novel compound, NA808, which is a derivative of the previously described compound NA255 with further improved properties, including improved replicon potency from a 50% effective concentration of 2 nM for NA255 to a 50% effective concentration of 0.84 nM for NA808.[12]

Here, we report the effectiveness of NA808 alone and in combination with DAAs. We used chimeric mice with humanized liver infected with HCV genotype 1a, 1b, 2a, 3a, and 4a to evaluate the potential of NA808 as a novel host-targeted HCV inhibitor.

Materials and Methods

Compounds

NA808 and telaprevir were synthesized by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). PEG-IFN was purchased from Chugai Pharmaceutical Co., Ltd. Non-nucleoside polymerase inhibitor, HCV-796, and nucleoside polymerase inhibitor, RO-9187,[13] were synthesized by F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

Development of Drug-Resistance Mutations in HuH7 Cells Harboring Subgenomic Replicon

The HCV subgenomic replicon cell line R6 FLR-N[14] (genotype 1b, HCV-N) was cultured with GlutaMax-I (DMEM-GlutaMax-I; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum in the presence of 0.5 mg/mL G418 and 48–72 nM NA808 or 1.8–2.7 μM telaprevir at a concentration of 4–6 times the 50% inhibitory concentration (IC_{50}) value for 14 passages. For the replicon assay, cells were seeded in 96-well tissue culture plates, and a 3-fold gradual dilution of NA808 or telaprevir in 5% fetal bovine serum supplemented GlutaMax-I was added. Serial dilutions of both compounds were prepared from the stock solutions dissolved in dimethyl sulfoxide at a concentration of 1 mM for NA808 and 50 mM for telaprevir. Luciferase activity was determined with a Steady-Glo luciferase assay kit (Promega, Madison, WI).

Deep Sequencing of HCV Genomes From Genotype 1b Replicon Cells and Genotype 1a–Infected Chimeric Mice

Deep sequencing of the HCV coding sequences was performed by using the GS Junior System (Roche Diagnostics, Mannheim, Germany), according to manufacturer's instructions. First, the acid guanidinium thiocyanate-phenol-chloroform extraction method was used to extract total RNA from the R6 FLR-N replicon cells after 14 passages with telaprevir or NA808 at a concentration of 6 times the IC_{50} value, or from the liver tissue of HCV-infected chimeric mice that were treated with or without NA808 for 14 days. Complementary DNA (cDNA) was then synthesized from the total RNA with random primers by using Superscript III Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA). The sequence of nucleotides 3429–9727 of the HCV genotype 1b replicon (R6NRz) genome or nucleotides 325–9381 of the HCV genotype 1a (HCG9) genome, including all of the HCV protein coding sequence, was divided into several segments of 1.5–3 kb with overlapping regions. Four segments of the genotype 1b replicon genome were amplified from the cDNA by polymerase chain reaction (PCR) with specific primers (Supplementary Table 2), and 7 segments of the genotype 1a (HCG9) genome were amplified from the cDNA by nested PCR with the indicated primers (Supplementary Table 3) by using PrimeSTAR GXL DNA Polymerase (TaKaRa Bio, Shiga, Japan). The amplified segments of HCV cDNA were purified from 1% agarose gels by using a MinElute Gel Extraction Kit (Qiagen, Valencia, CA) and quantified by measuring absorbance at 260 nm with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

The cDNA segments covering the coding sequence of HCV were then pooled together at approximately equimolar ratios. The Covaris S220 system (Covaris, Woburn, MA) was used to shear 500 ng of the pooled cDNA into 700- to 800-bp fragments. The sheared cDNA fragments were purified with the MinElute PCR Purification Kit (Qiagen), ligated with RL MID adaptors (Roche Diagnostics) to prepare the multiple cDNA libraries, and further purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The quality and quantity of the libraries were assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the KAPA Library Quantification Kit (Nippon Genetics, Tokyo, Japan), respectively. The libraries were then subjected to emulsion PCR, and enriched DNA beads (approximately 10% recovery) were loaded onto a picotiter plate and pyrosequenced with a GS Junior sequencer by using titanium chemistry (Roche Diagnostics). Several libraries derived from the HCV genomes generated by different treatments were sequenced in a single GS Junior run. The data obtained were analyzed by the GS Reference Mapper software (Roche Diagnostics) to identify resistant mutations.

SPT Assay

Crude extracts of the HCV subgenomic replicon cell line FLR3-1[12] (genotype 1b, Con-1) were used as a source of SPT in this assay. Briefly, FLR3-1 cells were suspended in HSS buffer (10 mM HEPES-KOH, 25 mM sucrose, and 0.1% sucrose monolaurate) containing 1/100 volume of protease inhibitor cocktail (Sigma, St Louis, MO) and sonicated 10 times with short pulses. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was stored at −80°C until use. Crude extract of FLR3-1 cells was added to 0.015 mL of a reaction mixture containing 200 mM HEPES buffer (pH 8.0), 5 mM EDTA, 10 mM dithiothreitol, 0.05 mM pyridoxal 5-phosphate, 0.05 mM palmitoyl-CoA, and 0.06 mM L-[^{14}C]serine in the presence of NA808. After a 15-minute incubation at 37°C, 0.3 mL chloroform/methanol (1:2, v/v), 0.1 mL phosphate-buffered saline, and 0.1 mL chloroform were added and mixed well. The extracts were

spotted on TLC plates and chromatographed. Radioactive spots were evaluated by using a Bio-imager.

Infection of HCV Genotype 1a, 1b, 2a, 3a, and 4a in Chimeric Mice With Humanized Liver

Chimeric mice were purchased from PhoenixBio Co., Ltd. (Hiroshima, Japan). The mice were generated by transplanting human primary hepatocytes into severe combined immunodeficient mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter (*Alb-uPA*). HCG9 (genotype 1a, GenBank accession number AB520610), HCR6 (genotype 1b, AY045702), HCR24 (genotype 2a, AY746460), HCV-TYMM (genotype 3a, AB792683), and HCVgenotype4a/KM (genotype 4a, AB795432) were intravenously injected into the chimeric mice with humanized liver at 10^4 (for HCR6, HCR24, HCV-TYMM, and HCVgenotype4a/KM) or 10^6 (for HCR6 and HCG9) copies/mouse. After 4 weeks, the HCV RNA levels in the mice sera had reached approximately 10^8 copies/mL for HCG9 and HCV-TYMM and approximately 10^7 copies/mL for HCR6, HCR24, and HCVgenotype4a/KM. The protocols for animal experiments were approved by our institutional ethics committee. The animals received humane care according to National Institutes of Health guidelines. Patients gave written informed consent before collection of blood or tissue samples.

Administration of NA808 and/or PEG-IFN, Telaprevir, HCV-796, RO-9187 into HCV-Infected Chimeric Mice With Humanized Liver

Treatment was started 12 weeks after HCV inoculation and continued for 14 days. Each treatment group contained at least 3 animals. NA808, PEG-IFN, RO-9187, HCV-796, and telaprevir were administered alone or in combination to chimeric mice infected with HCV genotype 1a (HCG9), genotype 1b (HCR6), genotype 2a (HCR24), genotype 3a (HCV-TYMM), or genotype 4a (HCVgenotype4a/KM). Blood samples and liver samples were collected according to the protocols shown in Supplementary Table 1. All DAAs were used at suboptimal doses to allow the demonstration of synergy when administered in combination therapy.

Quantification of HCV RNA by Real-Time Reverse Transcription PCR

Total RNA was purified from 1 μL chimeric mouse serum by using SepaGene RV-R (Sanko Junyaku Co., Ltd., Tokyo, Japan) and total RNA was prepared from liver tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method. HCV RNA was quantified by quantitative real-time PCR using techniques reported previously.[15] This technique has a lower limit of detection of approximately 4000 copies/mL for serum. Therefore, all samples in which HCV RNA was undetectable were assigned this minimum value.

Statistical Analysis

Statistical analysis was performed using the Student *t* test. A P value $<.05$ was considered statistically significant.

Results

In Vitro Characteristics of NA808

NA808 (Figure 1*A*), a derivative of NA255 isolated from fungal metabolites of *Fusarium incarnatum* F1476 demonstrated potent antiviral activity in HCV genotype 1b replicon cells with no apparent cellular toxicity under the assay conditions (Supplementary Figure 1*A*) and decreased HCV propagation in genotype 2a HCVcc-producing cells (Supplementary Figure 1*C*). NA255 is a selective inhibitor of SPT that inhibits HCV replication by suppressing the biosynthesis of sphingolipids that are required for HCV replication in replicon cells.[12] NA808 also inhibited the de novo synthesis of sphingolipids (Supplementary Figure 1*B*). According to the resulting Lineweaver-Burk plot of SPT inhibition in a replicon cell lysate, NA808 exhibited a noncompetitive inhibition pattern (Figure 1*B*). These findings suggest that NA808 inhibits HCV replication activities through the prevention of sphingolipid biosynthesis by a noncompetitive inhibition mechanism of SPT.

NA808 Shows a High Barrier to Resistance In Vitro

To evaluate the potential development of resistance to NA808, replicon cells (R6 FLR-N) were cultured in the presence of both G418 and NA808 at a concentration of 4 to 6 times the IC_{50} for 14 passages. Obvious changes in drug sensitivities to NA808 were not observed in these continuously treated replicon cells (Figure 2*A*), and the IC_{50} values were 18.9 nM (no treatment), 14.3 nM (treatment with 4 times the IC_{50}), and 19.8 nM (treatment with 6 times the IC_{50}). In contrast, there was a 5- to 17-fold increase of the IC_{50} values for telaprevir, an NS3/4 serine protease inhibitor, in replicon cells treated with 4 to 6 times the IC_{50} of telaprevir for the same duration (Table 1). The coding sequences of NS3 to NS5B from the replicon system after 14 passages with telaprevir or NA808 were determined by using deep sequencing. The sequences obtained at the 14th passage with telaprevir contained 3 known protease inhibitor resistance mutations (V36A, T54V, and A156T)[16] and NS5 region (Q181H, P223S, and S417P) (Table 2), suggesting that the increase in IC_{50} with telaprevir was accompanied by a shift in viral sequence. In contrast, no significant mutations were found in the 14th passage with NA808. Continuously treated replicon cells developed resistance to telaprevir, but not to NA808.

Anti-HCV Activities of NA808 in Chimeric Mice With Humanized Liver Infected With HCV

To evaluate the anti-HCV effect of NA808 in vivo, we used chimeric mice with humanized liver infected with HCV genotype 1a (HCG9) or 1b (HCR6). The chimeric mice with humanized liver were immunodeficient transgenic uPA/severe combined immunodeficient mice with reconstituted human liver; this mouse model supports long-term HCV infections at clinically relevant titers.

We administered NA808 via intravenous injection according to the schedule shown in Supplementary Table 1. In mice infected with HCV genotype 1a, NA808 (5 mg/kg/d) led to a rapid decrease in serum HCV-RNA (approximately a

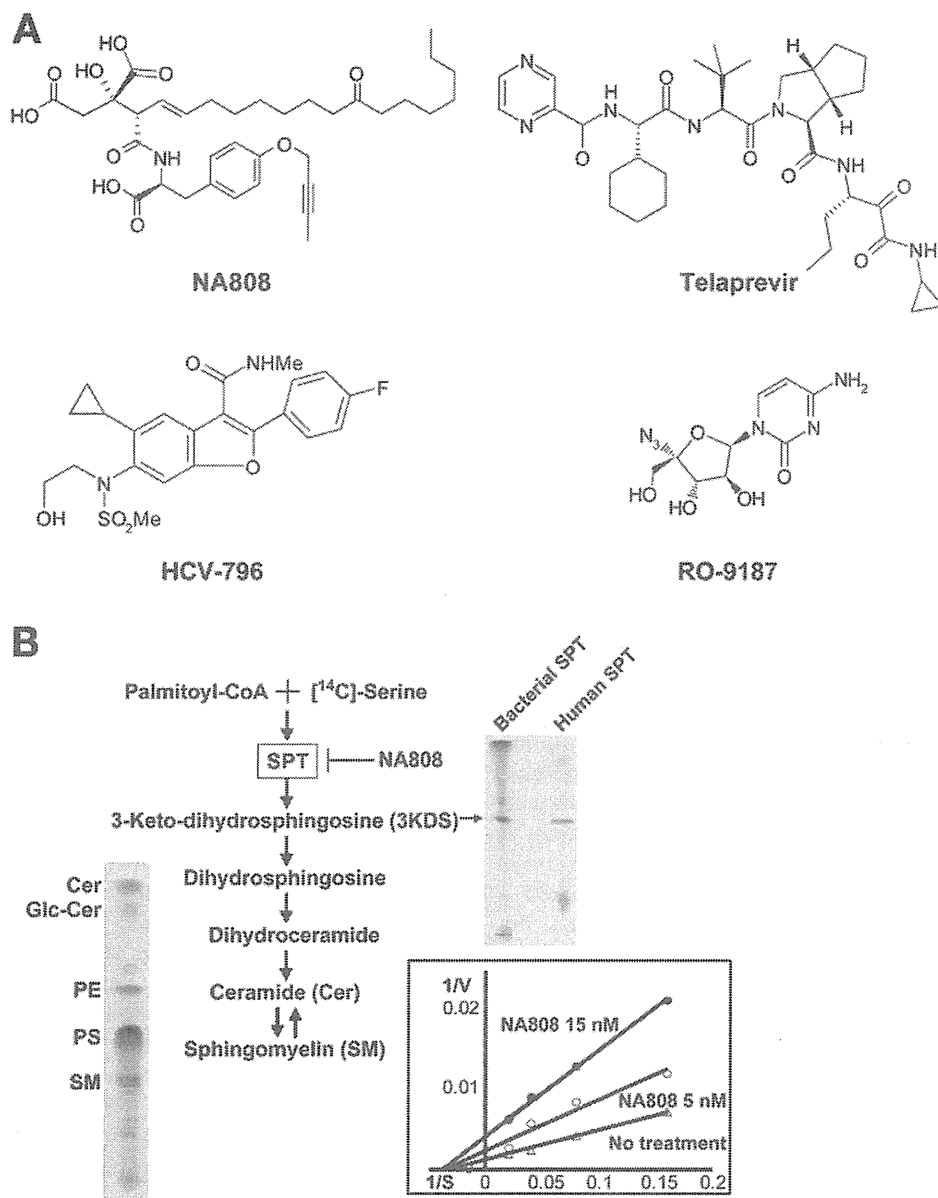

Figure 1. Characteristics of NA808. (*A*) Chemical structures of the compounds used in this study. (*B*) Scheme of de novo sphingolipid biosynthesis and Lineweaver-Burk plot of SPT assay results. Crude extract of FLR3-1 cells was incubated with L-[^{14}C]serine in the presence of NA808. After incubation at 37°C, the extracts were spotted on TLC plates and chromatographed. 3-Keto-dihydrosphingosine (3-KDS) generated from a bacterial SPT reaction is shown as a positive control marker.

2-log decrease within 14 days) (Figure 2*B*). A similar decrease in serum HCV-RNA occurred in mice infected with HCV genotype 1b that were treated with NA808 (5 mg/kg/d) (Figure 2*D*). NA808 also reduced hepatic HCV-RNA at the end of the treatment period in a dose-dependent manner (Figure 2*C* and *E*). These results indicate that NA808 has a robust antiviral effect in chimeric mice with humanized liver infected with HCV genotype 1a or 1b. The most effective dose was 5 mg/kg/d in both genotype 1a– and genotype 1b–infected mice; therefore, we used this dose for further experiments. To address whether NA808 had antiviral activity across HCV genotypes, chimeric mice infected with various strains of HCV were treated with 5 mg/kg of NA808 for 14 days, and then the HCV-RNA levels in the sera were evaluated. Inoculation with several HCV strains, HCG9 (genotype 1a), HCR6 (1b), HCR24 (2a), HCV-TYMM (3a), and HCVgenotype4a/KM (4a), resulted in HCV titers in the sera of mice of approximately 10^8 (HCG9 and HCV-TYMM) and 10^7 (HCR6, HCR24 and HCVgenotype4a/KM) copies/mL, respectively (Supplementary Figure 2, and as described previously[17]). At 14 days after administration, NA808 treatment resulted in approximately 1- to 3-log reductions of serum HCV-RNA in each genotype-infected group (Figure 2*F*). Human serum albumin levels were not changed during the administration period (data not shown), suggesting that the anti-HCV activity of NA808 against several genotypes occurred without any overt toxicity. NA808 was effective and well tolerated in chimeric mice with humanized liver infected with several genotypes of HCV.

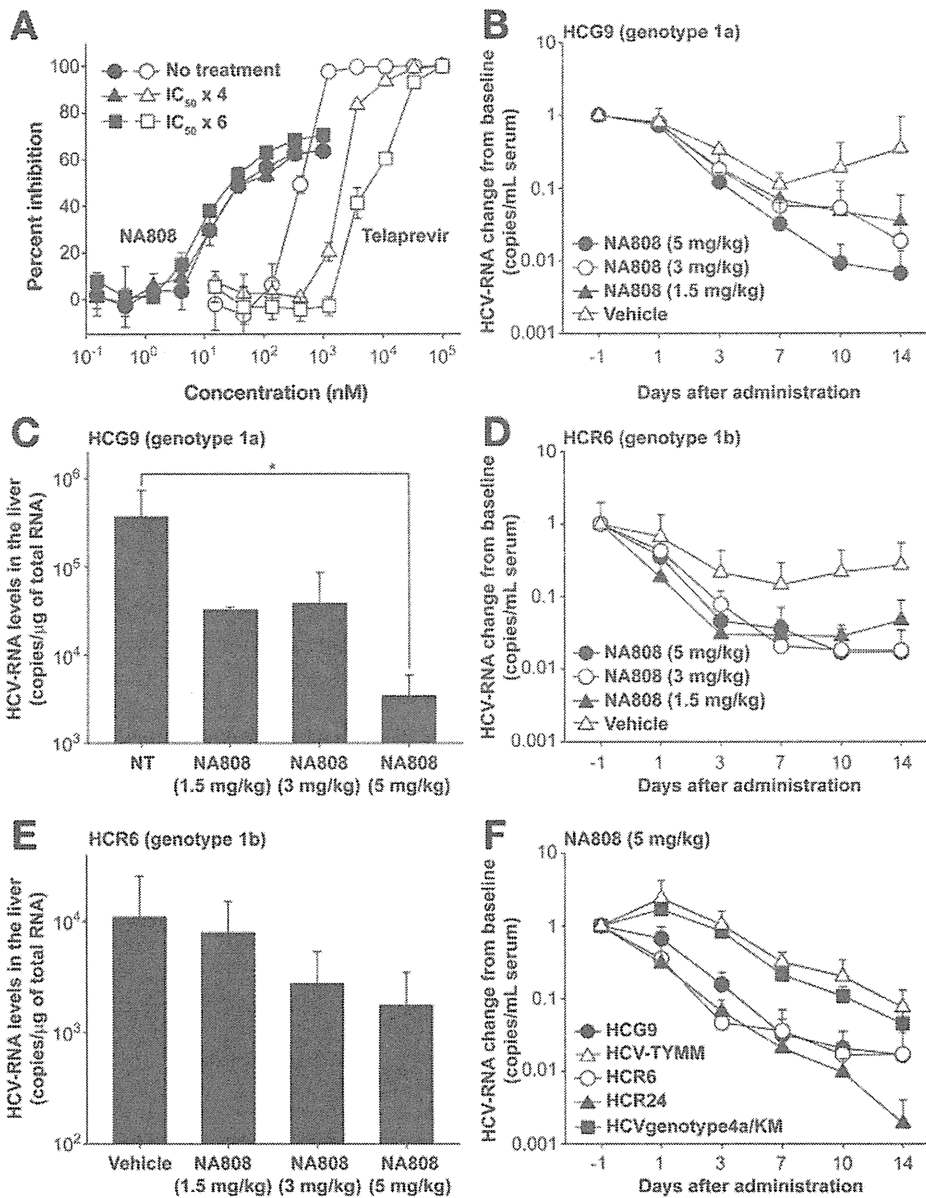

Figure 2. Drug-resistance profile and antiviral effects of NA808 in various HCV genotypes. (*A*) Activities on replicon cells cultured with NA808. Drug-resistant HCV replicons were selected in the presence of G418 and NA808 at a concentration of 4 to 6 times the IC_{50}. Changes in drug sensitivity were examined after the 14th passage. (*B, D*) Time course of serum HCV-RNA levels in chimeric mice infected with HCV genotype 1a (B) or genotype 1b (*D*) treated with vehicle or several doses of NA808 (*closed circles*: 5 mg/kg/d; *open circles*: 3 mg/kg/d; *closed triangles*: 1.5 mg/kg/d; *open triangles*: vehicle). (*C, E*) HCV-RNA levels in the livers of chimeric mice infected with HCV genotype 1a (*C*) or genotype 1b (*E*) at the end of treatment. Error bars = SD (*$P < .05$). (*F*) Time course of serum HCV-RNA levels in the sera of genotype 1a (*closed circles*), 1b (*open circles*), 2a (*closed triangles*), 3a (*open triangle*), and 4a (*closed squares*) after intravenous administration of 5 mg/kg/d NA808. Error bars = SD.

Deep Sequencing of HCV Genotype 1a From Chimeric Mice With Humanized Liver

Full-genome sequence analysis of HCV in the humanized-liver mouse model after 14 days of NA808 administration was performed. The viral RNA was extracted from liver tissues of humanized-liver mice, amplified by using the primer sets shown in Supplementary Table 3 and sequenced with the Roche/454 GS Junior sequencer by using titanium chemistry. We obtained 43,911 and 68,272 sequence reads for HCV genomes from untreated mice and from NA808-treated mice, respectively. The sequences were determined by comparing with the HCG9 reference sequence (GenBank accession number AB520610). As a result, the viral sequences from NA808-treated mice were identical to those from untreated mice.

Table 1. Changes in Drug Sensitivities of HCV Replicon Cells After the 14th Passage in the Presence of NA808 or Telaprevir

Drug	No treatment	IC_{50}×4	IC_{50}×6
NA808 (*nM*)	18.9 ± 2.82	14.3 ± 5.52	19.8 ± 7.86
Telaprevir (*μM*)	0.39 ± 0.022	2.14 ± 0.019	6.48 ± 1.30

Data are indicated as mean ± SD.

Synergistic Effects of NA808 With PEG-IFN or DAAs in Chimeric Mice Infected With HCV

The in vivo synergistic effects of NA808 combined with PEG-IFN on HCV replication were investigated by using chimeric mice with humanized liver infected with HCV genotypes 1a, 2a, and 4a. NA808 was administered