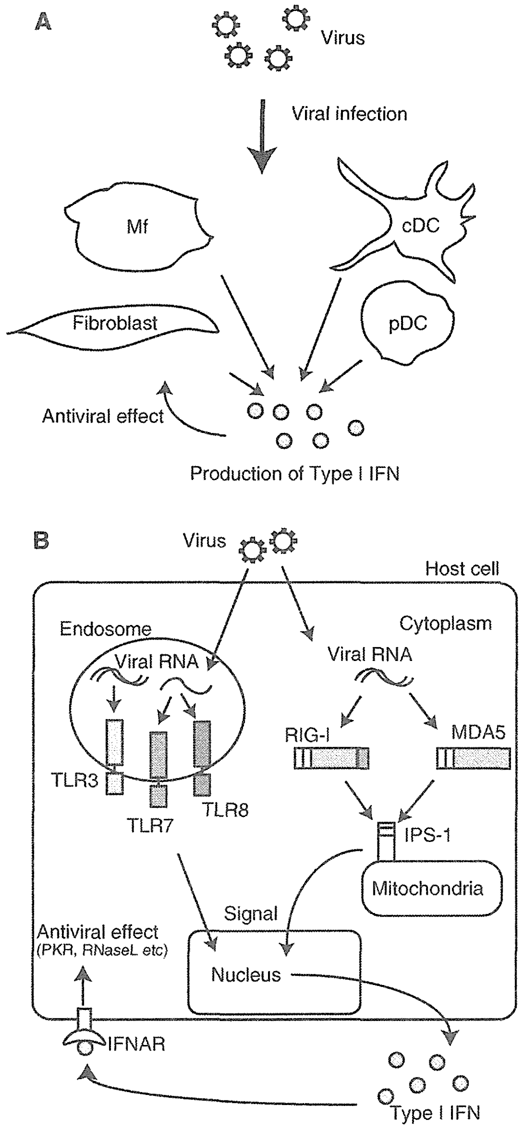

Fig. 1 Production of type I IFN in response to viral infection. (A) Type I IFN is a cytokine that possesses strong anti-viral activity. Type I IFN is produced from fibroblast cells, cDC, pDC and Mf in response to viral infection. (B) TLR3, 7 and 8 are localized to endosomes and are responsible for the recognition of viral RNA. Viral RNA in the cytoplasmic region is recognized by RIG-I and MDA5, leading to the activation of the adaptor molecule IPS-1. IPS-1 triggers the signal to induce type I IFNs. Type I IFNs binds to an IFN receptor, IFNAR, leading to the activation of anti-viral factors, such as PKR and RNaseL.

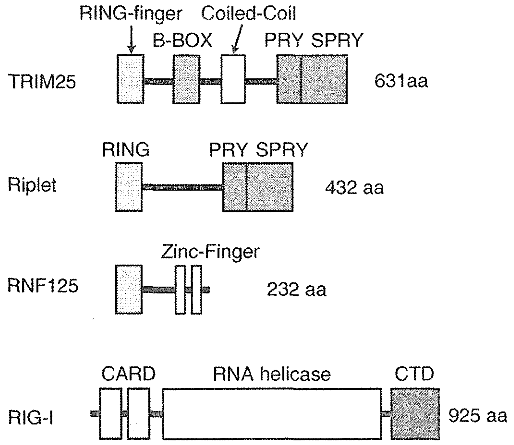

Fig. 2 Domain structures of TRIM25, Riplet, RNF125 and RIG-I. TRIM25 consists of RING finger, B-box, coiled-coil, PRY and SPRY domains. Riplet is similar to TRIM25 and consists of RING-finger, PRY and SPRY domains. RNF125 consists of RING-finger and two zinc-finger domains. Three proteins mediate the polyubiquitination of RIG-I. RIG-I consists of two N-terminal CARDs, central RNA helicase and CTDs.

mortality due to viral infections (*2*, *19*). Thus, RIG-I-dependent pathways are necessary for efficient early type I IFN production and are required for protection against viral infections (*18*).

TRIM25 ubiquitin ligase is a positive factor for the RIG-I activation

During viral infection, the RIG-I protein has a modified form of ubiquitin. TRIM25 (also called Efp) is a ubiquitin ligase (*22*, *23*), and its domain structure is described in Fig. 2. This protein interacts with the first CARD of RIG-I (*22*, *24*). T55I mutation of the first CARD of RIG-I is found in RIG-I-deficient HuH7.5 cells. T55 of RIG-I is critical for the interaction between TRIM25 and RIG-I (*9*, *24*, *25*). Gack et al. detected the polyubiquitination of the K99, K169, K172, K181, K190 and K193 residues of RIG-I CARDs by mass spectrometry analysis (*22*), and the K172R mutation alone causes a near-complete loss of the polyubiquitination of RIG-I CARDs (*22*). TRIM25 delivers the K63-linked polyubiquitin moiety to the K172 residue of the second CARD of RIG-I, leading to efficient interaction with IPS-1/MAVS/VISA/Cardif (*22*, *24*). On the other hand, Zeng *et al.* reported another mechanism of the activation of RIG-I by ubiquitin. They reconstituted RIG-I pathway *in vitro* and showed that RIG-I CARDs sense unanchored polyubiquitin chains mediated by TRIM25, and the binding of RIG-I CARDs to the unanchored polyubiquitin chains leads to the activation of RIG-I (*26*). Knockout of TRIM25 abrogates IFN-β production from MEF in response to viral infection (*22*). Thus, ubiquitination or polyubiquitin binding is essential for the activation of RIG-I (Fig. 2).

The expression of a splice variant of RIG-I mRNA is robustly up-regulated upon viral infection (*24*). This splice variant encodes a protein that lacks the first 36–80 amino acid region within the first CARD of RIG-I; therefore, the RIG-I splice variant (RIG-I SV) protein loses TRIM25 binding, CARD ubiquitination and downstream signalling ability (Fig. 3) (*24*). RIG-I SV inhibits the multimerization of the wild-type RIG-I protein and IPS-1 interaction and shows a dominant negative effect on the RIG-I-mediated anti-viral IFN response (*24*). Thus, RIG-I SV acts as the off switch regulator of its own signalling pathway (*24*).

In addition to the IPS-1 adaptor molecule, RIG-I also binds to the inflammasome adaptor apoptosis-associated speck-like protein containing a CARD domain (ASC), also known as Pycard, in response to viral infection (*27*). ASC activates caspase-1, leading to the proteolytic processing of pro-IL-1β into mature, bioactive IL-1β (*28*). TRIM25 activity is dispensable for caspase-1 activation through ASC (*27*). Thus, RIG-I polyubiquitination by TRIM25 is dispensable for ASC inflammasome adaptor activation (*27*).

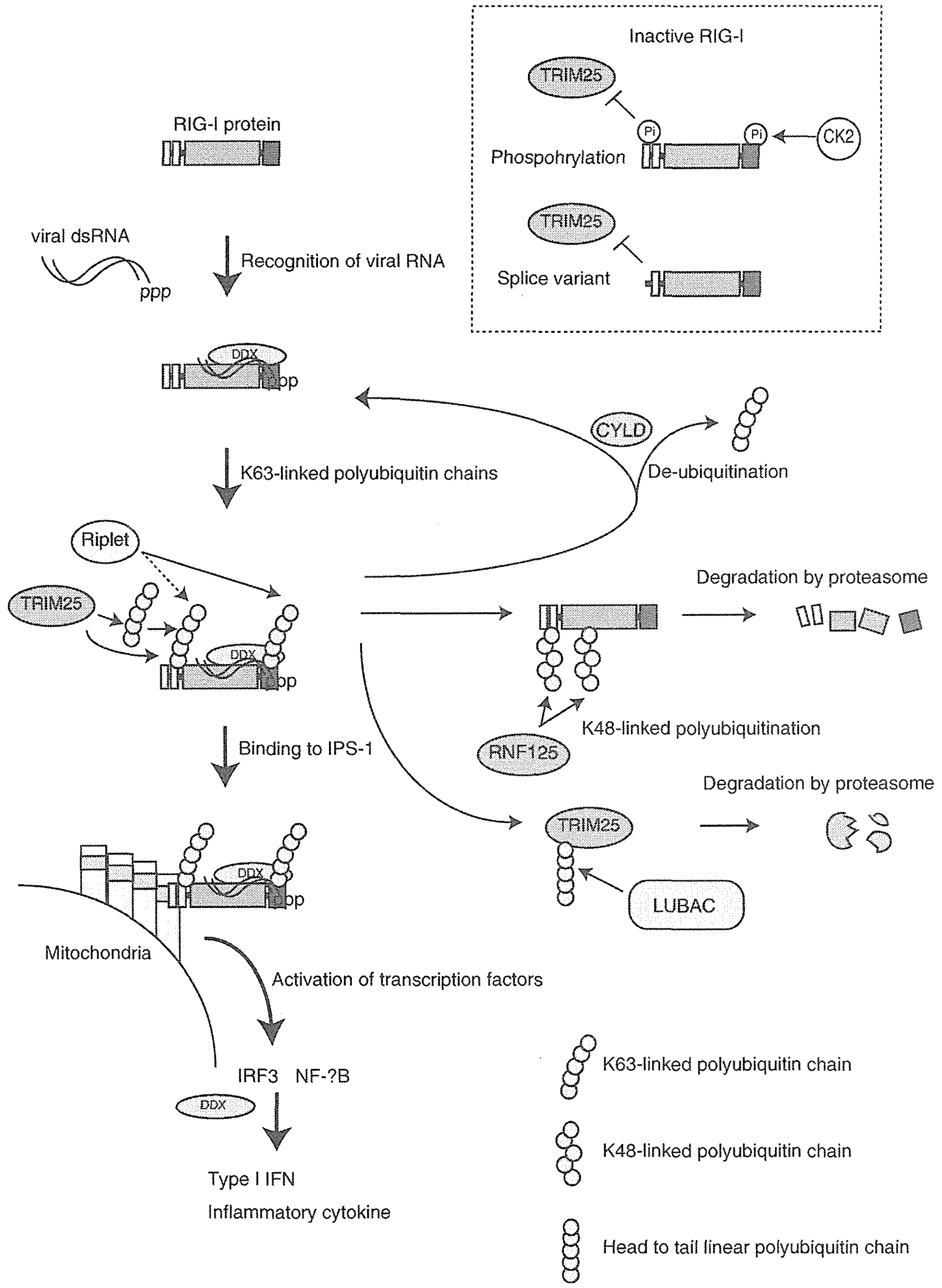

Fig. 3 Regulation of RIG-I by the ubiquitin chain. RIG-I binds to viral RNA together with other cofactors, such as DDX3. After the recognition of viral RNA, RIG-I changes its conformation and harbours K63-linked polyubiquitination by TRIM25 and Riplet. Polyubiquitination causes the activation of IPS-1, leading to the production of type I IFN. CYLD, a deubiquitin enzyme, removes the polyubiquitin chain of RIG-I. CK2 and other unknown kinase phosphorylate RIG-I, and the phosphorylated RIG-I protein is not polyubiquitinated by TRIM25. In addition, splice variant RIG-I (SV RIG-I) is not polyubiquitinated by TRIM25, and the SV RIG-I protein acts as a dominant negative form. RNF125 mediates the K48-linked polyubiquitinatioin of RIG-I, which causes the degradation of RIG-I by proteasomes. The LUBAC protein complex suppresses TRIM25 function by mediating the head-to-tail polyubiquitination of TRIM25.

However, RIG-I polyubiquitination is essential for NF-κB activation by RIG-I, which is required for IL-1β mRNA expression; thus, knockout of TRIM25 reduces the production of mature IL-1β (*4*, *19*, *27*).

Riplet ubiquitin ligase is essential for the activation of RIG-I

Riplet (also called Reul or RNF135) was isolated by yeast two-hybrid screening to isolate RIG-I CTD binding proteins (*29*). The Riplet protein is composed of N-terminal RING finger, C-terminal SPRY and PRY domains, and is similar to TRIM25 (Fig. 2). However, this protein lacks B-box, which is a typical feature of TRIM family proteins. Thus, the protein does not belong to the TRIM family. Riplet expression is observed in various tissues and cells such as DC, Mfs and MEF (*29*, *30*). Hu *et al.* (*31*) detected endogenous Riplet protein in human DC lysates. Riplet expression is induced in mouse bone marrow-derived DCs (BM-DCs) by polyIC stimulation, which is a double-stranded RNA analog; however, its expression is not changed in human fibroblast and HeLa cells (*29*).

The Riplet protein physically interacts with RIG-I CTD, and in some experimental conditions, it binds to RIG-I CARDs (*29*, *32*). The Riplet C-terminal region is responsible for this binding. Riplet mediates K63-linked polyubiquitination of RIG-I CTD, leading to the activation of RIG-I (Fig. 3) (*29*). The five CTD lysine residues at 849, 851, 888, 907 and 909 are important for the polyubiquitination and activation of RIG-I (*29*, *30*). In contrast, Gao *et al.* (*32*) reported that Riplet mediates K63-linked polyubiquitination of K154, K164 and K172 of RIG-I CARDs in their experimental conditions (Fig. 3).

In some strain backgrounds, RIG-I-deficient mice are embryonic lethal, but Riplet knockout mice are born at expected Mendelian ratios from Riplet$^{+/-}$ mice (*19*, *30*, *33*). Moreover, the development of DCs and Mfs is also normal in Riplet-deficient mice (*30*). Douglas *et al.* (*30*, *34*) reported that Riplet/RNF135 haploinsufficiency causes an overgrowth syndrome and learning disabilities in human; however, knockout of the Riplet gene in mice does not cause any apparent defects with regard to development. Knockout of Riplet severely reduces the production of type I IFN and abrogates the activation of RIG-I and RIG-I CTD polyubiquitination (*30*). Riplet knockout mice are more susceptible to VSV infection than wild-type mice. As IPS-1 and STING, Riplet is necessary for efficient, early type I IFN production *in vivo*, but it is dispensable for late type I IFN productions (*30*). This indicates the essential role that Riplet plays in the RIG-I-dependent innate immune response against RNA virus infection. Genetic evidence shows that knockout of either Riplet or TRIM25 destroyed the RIG-I-dependent innate immune response; therefore, both ubiquitin ligases are required for the activation of RIG-I in response to RNA virus infection (*22*, *30*). RLR pathways contribute to type I IFN expression in response to cytoplasmic DNA (*35–37*). However, Riplet-independent type I IFN expression pathway in response to cytoplasmic DNA exists in MEF (*30*).

Ubiquitin ligases target several proteins. For example, TRIM25 targets the proteolysis of 14-3-3 σ, a negative cell cycle regulator that causes G2 arrest, and thus, promotes breast tumour growth (*23*). Proteome analysis reveals that Riplet binds to the TRK-fused gene (TFG), which is a target of chromosome translocation in lymphoma (*38–40*). Pasmant *et al.* (*41*) reported that the Riplet/RNF135 gene is down-regulated in tumour Schwann cells from malignant peripheral nerve sheath tumours, and their study suggested the involvement of Riplet/RNF135 in an increased risk of malignancy observed in NF1 microdeletion patients. Thus, it is possible that Riplet targets not only RIG-I but also other proteins.

Negative regulators of RIG-I

The RNF125 (also called TRAC1) protein possesses a RING finger domain and functions as a ubiquitin ligase (*42*). Arimoto *et al.* (*43*) isolated RNF125 by yeast two-hybrid screening to obtain the protein that binds to UbcH8, which is an E2 ubiquitin-conjugating enzyme, and found that RNF125 also binds to RIG-I. Unlike Riplet and TRIM25, RNF125 ubiquitin ligase mediates K48-, but not K63-linked polyubiquitination of RIG-I, leading to the degradation of RIG-I by proteasomes (Fig. 3) (*43*). UbcH5c is possibly an E2 enzyme, which cooperates with RNF125, and UbcH8 acts as a negative factor in the RNF125-mediated polyubiquitination of RIG-I (*43*, *44*). Furthermore, RNF125 ubiquitinates MDA5, a member of RLRs, and the expression of RNF125 impairs MDA5-mediated signalling (*43*). RNF125 expression is induced by type I IFN and polyIC treatment. The increase in RNF125 mRNA expression correlates temporally with the decrease in RIG-I expression (*43*). Knockdown of RNF125 increases the type I IFN expression in response to viral infection (*43*). Since RNF125 is enhanced by type I IFN, the function of RNF125 constitutes a negative regulatory loop circuit for type I IFN production.

CYLD is a deubiquitinase that cleaves the K63-linked polyubiquitin chain. This protein acts as a negative regulator of NF-κB and Jun N-terminal kinase signalling pathways by cleaving the K63-linked polyubiquitin chains of NEMO, TRAF2 and BCL3 (*45–48*). Friedman *et al.* (*49*) performed a microarray analysis and found that the expression profile of RIG-I is correlated with that of CYLD. Moreover, they found that the CYLD protein physically interacts with RIG-I, TBK1 and IKKε, and deubiquitinates these proteins. CYLD inhibits SeV-induced type I IFN production. Thus, it is expected that CYLD attenuates the establishment of an anti-viral state (Fig. 3).

There are host and viral negative regulators for TRIM25. HOIL-1L and HOIP are members of the RING-IBR-RING (RBR) E3 ubiquitin ligase family and form complexes (*50*). HOIL-1L and HOIP form ubiquitin polymers through the linkage between the C- and N-termini of the ubiquitin molecules in order to assemble a head-to-tail linear polyubiquitin chain; thus, the protein complex is designated as LUBAC (linear ubiquitin assembly complex) (*50*). LUBAC has the ability to induce polyubiquitination of TRIM25; it specifically suppresses TRIM25-mediated RIG-I ubiquitination by inducing TRIM25 degradation and inhibiting TRIM25 interaction with RIG-I (Fig. 3) (*51*). Excessive production of IFNs or inflammatory cytokines is destructive rather than protective; thus, an absolute regulation of the immune signalling pathway is essential for a successful immune response against viral infections. HOIL-1L- and HOIP-mediated suppression of TRIM25 would be important for the absolute regulation of an immune response (*51*).

Viruses have evolved sophisticated mechanisms to evade the host IFN system. There are several virus-encoded IFN antagonists that inhibit host innate anti-viral responses. NS1 of the influenza A virus is one of the IFN antagonists (*52*, *53*). It sequestrates viral dsRNA from cellular sensors including RIG-I (*52*). In addition, it interacts with the coiled-coil region of TRIM25 and blocks TRIM25 multimerization and RIG-I CARD polyubiquitination (*54*).

Perspectives

Several ubiquitin-like proteins (UBLs) exist. ISG15 is a UBL and is induced in response to viral infection (*55*). Several anti-viral proteins are modified by ISG15, including RIG-I (*44*, *55*). UbcH8 is an E2 enzyme that promotes ISG15 conjugation to RIG-I (*44*). However, ISG15 knockout mice do not either reduce immunological functions or decrease anti-viral activity (*56*). Thus, the physiological role of ISG15 conjugation to RIG-I remains unknown.

In addition, the RIG-I protein is modified by phosphorylation. The T170 residue of RIG-I is phosphorylated under normal conditions, and phosphorylation is reduced after SeV infection (*24*). Phosphorylation of RIG-I CARDs inhibits the TRIM25-mediated polyubiquitination (Fig. 3). Thus, Gack *et al.* suggested that dephosphorylation of RIG-I permits the TRIM25 binding and TRIM25-mediated polyubiquitination of RIG-I, allowing RIG-I to form a stable complex with IPS-1 in order to trigger an IFN-mediated anti-viral innate immune response. However, the kinase and phosphatase that target RIG-I N-terminal CARDs are still unknown. In addition to RIG-I CARDs, RIG-I CTD is regulated by phosphorylation. In resting cells, casein kinase II (CK2) phosphorylates T770, and S854 and S855 (*57*). The phosphorylation of RIG-I CTD suppresses the RIG-I-mediated signalling (Fig. 3) (*57*). Following viral infection, phosphatases cause dephosphorylation of the RIG-I CTD, leading to the activation of RIG-I-mediated signalling (*57*).

RIG-I requires several cofactors. High mobility group box proteins are required for the RIG-I to recognize viral RNA (*58*). DDX3 and DDX60 are non-RLR helicases that are involved in RLR signallings, and play pivotal roles in RIG-I-mediated signalling (Fig. 3) (*59*–*62*). It remains to be determined whether the post-translational modification of RIG-I affects the interaction with those co-factors.

Riplet ubiquitinates RIG-I CTD. The molecular mechanism of how the Riplet-dependent polyubiquitination of RIG-I CTD triggers the downstream signalling remains to be determined yet. RIG-I CTD has two functions. In the absence of viral RNA, RIG-I CTD suppresses the activation of RIG-I CARDs. Following viral infection, RIG-I CTD binds to viral RNA, leading to the conformational changes and ultimately removal of the suppression. It is possible that CTD polyubiquitination affects both functions of RIG-I CTD.

Funding

Grants-in-Aid from the Ministry of Education, Science, and Culture and the Ministry of Health, Labor and Welfare of Japan.

Conflict of Interest

None declared.

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Microbiol Immunol 2012; **56**: 1–9
doi:10.1111/j.1348-0421.2011.00403.x

REVIEW

In vitro models for analysis of the hepatitis C virus life cycle

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ABSTRACT

Chronic hepatitis C virus (HCV) infection affects approximately 170 million people worldwide. HCV infection is a major global health problem as it can be complicated with liver cirrhosis and hepatocellular carcinoma. So far, there is no vaccine available and the non-specific, interferon (IFN)-based treatments now in use have significant side-effects and are frequently ineffective, as only approximately 50% of treated patients with genotypes 1 and 4 demonstrate HCV clearance. The lack of suitable *in vitro* and *in vivo* models for the analysis of HCV infection has hampered elucidation of the HCV life cycle and the development of both protective and therapeutic strategies against HCV infection. The present review focuses on the progress made towards the establishment of such models.

Key words hepatitis C virus, HuH-7 cell, knockout mice, type I interferon.

Chronic HCV infection is a major cause of mortality and morbidity throughout the world, infecting approximately 3.1% of the world's population (1). Only a fraction of acutely infected individuals are able to clear the infection spontaneously, whereas approximately 80% of infected individuals develop a chronic infection (2, 3). Patients with chronic HCV are at increased risk for developing liver fibrosis, cirrhosis, and/or hepatocellular carcinoma. Currently, these long-term complications of chronic HCV infection are the leading indication for liver transplantation (4, 5). Because of the high incidence of new infections by blood transfusions in the 1980s before the discovery of the virus, and because morbidity associated with chronic HCV infection generally takes decades to develop, it is expected that the burden of disease in the near future will rise dramatically.

HCV is an enveloped flavivirus, with a positive-stranded RNA genome of approximately 9600 nucleotides. The coding region is flanked by 5′ and 3′ non-coding regions, which are important for the initiation of translation and regulation of genomic duplication, respectively. The coding region itself is composed of a single open reading frame, which encodes a polyprotein precursor of approximately 3000 amino acids. This polyprotein is cleaved by host and viral proteases into structural and NS proteins (Fig. 1). Replication of the HCV genome involves the synthesis of a full-length negative-stranded RNA intermediate, which in turn provides a template for the de novo production of positive-stranded RNA. Both these synthesis steps are mediated by the viral RNA-dependent RNA polymerase NS5B (6–8). NS5B lacks proofreading abilities, and this leads to a high mutation rate and the

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Received 8 September 2011; revised 23 October 2011; accepted 3 November 2011.

List of Abbreviations: 3-D, three-dimensional; 3-D/HF, three-dimensional hollow fiber system; bbHCV, blood borne hepatitis C virus; HCV, hepatitis C virus; HPV/E6E7, human papilloma virus E6/E7 genes; IFN, interferon; IFNAR, interferon A receptor; IRES, internal ribosome entry site; ko, knockout; MDA-5, melanoma differentiation associated gene 5; MEF, mouse embryo fibroblasts; mir199, micro RNA 199; NS proteins, non-structural proteins; PPAR, peroxisome proliferator-activated receptor; RFB, radial flow bioreactor; RIG-I, retinoic acid-inducible gene I; TLR, Toll-like receptor; uPA, urokinase plasminogen activator.

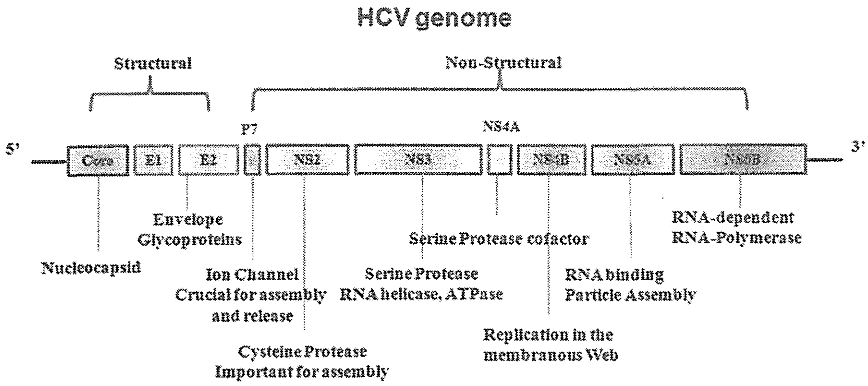

Fig. 1. Genomic structure of HCV. Genomic organization of wild-type HCV. The HCV-RNA genome consists of a major open reading frame, encoding a single polyprotein, and an alternative reading frame encoding F-proteins with unknown functions. The cleavage of the polyprotein by viral and host cell proteases gives rise to the mature structural (core, envelope proteins E1 and E2, and p7) and NS viral proteins (NS2 through NS5B). The putative activities and functions of viral proteins are indicated. The IRES located in the 5′ non-coding region initiates ribosome binding and translation. Both the 5′ and 3′ non-coding regions are essential for viral RNA replication involving the RNA-dependent RNA polymerase NS5B. NTPase, nucleotide triphosphatase.

generation of numerous quasispecies. HCV isolates can be classified into seven major genotypes, which vary in sequence by more than 30%. In addition to the distinct prevalence and global spread of the virus, the genotype is an important factor determining disease progression and responses to antiviral therapy (9).

Currently, the only licensed treatment for HCV is the combination of (pegylated)-interferon-alpha (IFN-α) and ribavirin. Although the success rate of treatment has improved substantially, standard therapy is not effective in all patients. Moreover, severe adverse effects and high costs limit the compliance and global application of this treatment. The development of prophylaxis and novel therapeutics to treat HCV infection has been hampered by the lack of suitable *in vitro* and *in vivo* culture systems. In this review, we describe the development of *in vitro* culture systems for HCV.

Tissue culture-adapted HCV (sub-)genomic replicons

Dr Bartenschlager's group was the first to establish a convenient reproducible *in vitro* cell culture system for the study of HCV replication (10). They created antibiotic-resistant HCV genomes to select replication-competent viral clones by conveying antibiotic resistance to cells. This was achieved by replacing the structural protein-coding sequences, as well as p7 of the consensus genome Con1, by the neomycin resistance gene. In addition, a second IRES was introduced to promote translation of the non-structural protein-coding sequences important for viral replication (Fig. 2). Upon transfection of these so-called subgenomic replicons in specific cell lines, drug-resistant cell colonies were isolated in which high levels of viral replication occurred. Subsequent analysis confirmed that these HCV replicons indeed were capable of self-amplification through synthesis of a negative-strand replication intermediate, and could be stably propagated in cell culture for many years (10, 11).

HCV replication was supported by several cell types such as HuH6 (12), HepG2 (13), Li23 (14), and 293 cells (15), with the human hepatoma cell line HuH-7 being the most permissive (16). Interestingly, removal of replicon RNA from these cell clones by treatment with type 1 IFN rendered the cells more permissive to reintroduction of replicons, resulting in higher replication rates. Examples of these highly permissive cells are HuH-7.5 and HuH-7-Lunet cells (16, 17). The efficient replication in the replicon systems was found to depend on tissue-culture-adaptive mutations. Introduction of these specific mutations in the wild-type consensus sequence significantly enhanced viral replication *in vitro* (18–22). Mutational hot spots were found clustered primarily in the NS3, NS4B, and NS5A regions. The mechanisms behind the enhanced replication caused by these tissue-culture-adaptive mutations are still largely unknown, and the interesting fact that these mutations are not commonly found in patients suggests that these may have a toll on the viral fitness.

HCV replicons have proven to be extremely valuable for studies on the process of HCV replication, as well as for testing novel antiviral compounds that specifically target the protease activity of NS3 or the polymerase activity of NS5 (23).

Cell culture-derived infectious HCV

Studies using HCV replicons have provided detailed knowledge on the mechanisms of replication of HCV.

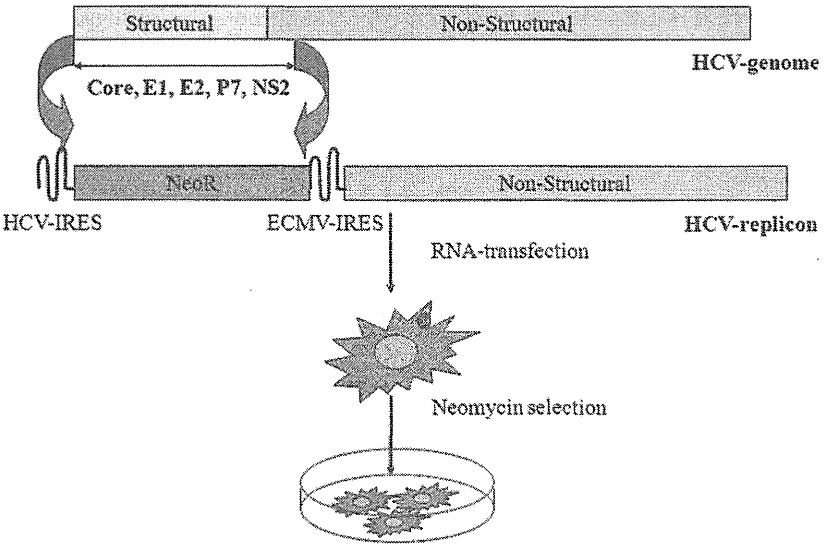

Fig. 2. HCV replicon system. The structural sequences (C, E1, E2, and p7) together with NS2 were replaced by a neomycin antibiotic-resistance gene, and an ECMV-IRES was introduced to drive translation of the remaining non-structural proteins. Neomycin selection of these double cistron (bicistronic) replicons in the hepatoma cell line Huh7 resulted in high-level HCV-RNA replication, depending on the gain of so-called 'tissue-culture' adaptive mutations mostly confined to the NS3, NS4B, and NS5A regions.

However, an apparent shortcoming of these models was that stable cell clones containing self-replicating replicons and expressing all viral proteins remained unable to release infectious HCV particles. The inability to secrete viral particles may be the consequence of adaptive mutations, which are needed to enhance viral replication rates, but at the same time may block viral assembly. Indeed, replicons without adaptive mutations show very low replication rates (16, 24). A different situation emerged when the first genotype 2a consensus genome was established (25, 26).

A subgenomic replicon constructed from a clone called JFH-1, isolated from a Japanese patient with fulminant hepatitis C, replicated up to 20-fold higher in HuH-7 cells as compared to Con1 replicons, and did not require adaptive mutations for efficient replication *in vitro* (26). Transfection of HuH-7 and HuH-7.5.1 cells with the *in vitro*-transcribed full-length JFH-1 genome or a recombinant chimeric genome with another genotype 2a isolate, J6, resulted in the secretion of viral particles that were infectious in cultured cells (Fig. 3), in chimeric mice, and in chimpanzees (27–29).

The infectivity of cells could be neutralized with antibodies against the HCV entry receptor CD81, antibodies against E2, or immunoglobulins from chronically infected patients. Importantly, the replication of cell-cultured HCV in this system was inhibited by IFN-α as well as by several HCV-specific antiviral compounds (29). Since 2005, chimeric JFH-1-based genomes have been constructed of all seven known HCV genotypes. Similar to the J6-JFH-1 chimera, in these so-called intergenotypic recombinants, the structural genes (core, E1, and E2), p7, and NS2 of JFH-1 were replaced by genotype-specific sequences which often resulted in lower infectious virion production than

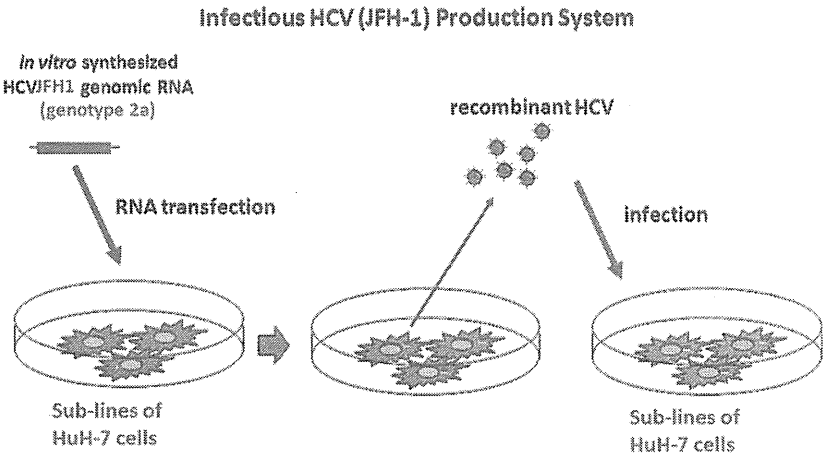

Fig. 3. JFH1 infectious system. Full-length JFH1-RNA is transcribed *in vitro*, and transfected to HuH-7-derived cell lines. JFH1 replicates in these cells, and produce infectious virions in the medium. The medium is collected, concentrated, and used to infect naive cells. Hence, the entire HCV life cycle was reproduced for the first time *in vitro*.

wild-type JFH-1 (30–32). Most NS proteins of intergenotypic chimeras originate from JFH-1, and therefore these genomes are unlikely to reflect genotype-specific characteristics of replication. However, these intergenotypic chimeras may become critically important in the study of differences in HCV entry or to assess the efficacy of HCV entry inhibitors. Interestingly, production of infectious genotype 1a HCV in cells transfected with synthetic RNA (H77-S) derived from a prototype virus (H77-C) was also reported (33). H77-S carries adaptive mutations that promote efficient viral RNA replication in HuH-7.5 cells. These mutations are located within the NS3/4A protease complex, and the NS5A protein (34) H77-S showed similar replication efficiency to JFH-1 isolate; however, it showed lower expression of HCV core protein, and lower production of infectious HCV particles (33).

Serum-derived HCV infection

The previously mentioned models used to study HCV infection are based on subclones of HuH-7 cells infected with JFH1 recombinant virus or its derivatives (27). HuH-7 cells and its subclones, however, do not support the entire life cycle of the bbHCV present in the blood of patients (35). Moreover, HCV has considerable diversity and variability. It is generally classified into six major genotypes and more than 100 subtypes (36). JFH1, however, is a single isolate of HCV genotype 2a that was originally derived from a patient with rare fulminant hepatitis (27). Thus, usage of HCV particles isolated from patient serum could be more useful to study authentic HCV infection.

Many researchers have attempted to develop an *in vitro* system for bbHCV (37–39). These current systems, however, are still insufficient due to their low efficiency for infectivity and replication of bbHCV. Normal human hepatocytes are the ideal system in which to study HCV infectivity. When cultured *in vitro*, however, they proliferate poorly and divide only a few times (40). Continuous proliferation could be achieved by introducing oncogenes, the HPV/E6E7 immortalized multiple cell types that were phenotypically and functionally similar to the parental cells (41–45). We established a human primary non-neoplastic hepatocyte cell line transduced with the HPV18/E6E7 that retained primary hepatocyte characteristics even after prolonged culture (35). We further improved the susceptibility of HPV18/E6E7-immortalized hepatocytes (HuS-E/2 cells) to bbHCV infectivity by impairing the innate immune response of these cells through suppression of interferon regulatory factor-7 (IRF-7) expression. These cells were useful to assay infectivity of HCV strains other than JFH-1, HCV replication, innate immune system engagement of HCV, and screening of anti-HCV agents. This infection system using non-neoplastic cells also suggested that IRF-7 plays an important role in eliminating HCV infection. Using this system, the suppressive effect of tamoxifen and mir199 on HCV replication was reported (46, 47).

Three-dimensional culture

A major limitation of the immortalized hepatocytes infection system was the failure to produce infectious HCV particles. Because the 3-D cell culture condition more closely reproduces the *in vivo* environment of hepatocytes (48), culturing these cells in this manner may support the entire HCV life cycle. Similarly, a previous report showed the production of HCV particles from the FLC4 hepatocyte line transfected with HCV-RNA and cultured in a 3-D radial-flow bioreactor (RFB). The RFB system is composed of a dedicated device containing 1 × 10^9 FLC4 cells with a culture area of 2.7 m^2. A more convenient, smaller and easy to use 3-D culture system is required for the study of the several aspects of bbHCV infection. (49). A hybrid artificial liver support system was developed using animal hepatocytes cultured in a 3-D/HF. This bioartificial liver showed several characteristic features of liver tissue for more than 4 months (50–52).

By growing our HuSE/2 cells in a similar 3-D culture (53) the gene expression profile was improved to more closely match that of human primary hepatocytes. We used this small 3-D culture system and showed it to be ideal for culturing HuS-E/2 cells for the study of bbHCV infection (Fig. 4) (54). Using this system we observed not only the enhancement of HCV replication, but also the production of infectious HCV particles in the medium using the 3-D/HF system. The cell mass formed by the 3-D culture system, most likely the polar character, was essential for the life cycle of bbHCV. Using microarray comparison of gene expression between 2-D and 3-D cultured cells, we found a higher activation of the PPAR-α signaling pathway which was shown to be important for the improvement of HCV replication in 3-D culture. Suppression of the PPAR-α signaling pathway using its antagonist MK886 markedly suppressed HCV replication in two different cell lines (53). A recent study showed that the induction of PPAR-α or PPAR-γ led to the suppression or enhancement of HCV replication, respectively, in HuH-7 cells (55). Using HuH-7-derived clones, three different independent studies confirmed our data, showing the suppression of HCV replication by PPAR-α blockers such as (MK886) (56, 57) or 2-chloro-5-nitro-*N*-(pyridyl) benzamide (BA) (58). Furthermore, no effect of PPAR-γ was observed on HCV replication (58).

Delayed production of infectious particles was also observed in cells infected with some HCV strains after prolonged culture (54). It is likely that mutation of the HCV

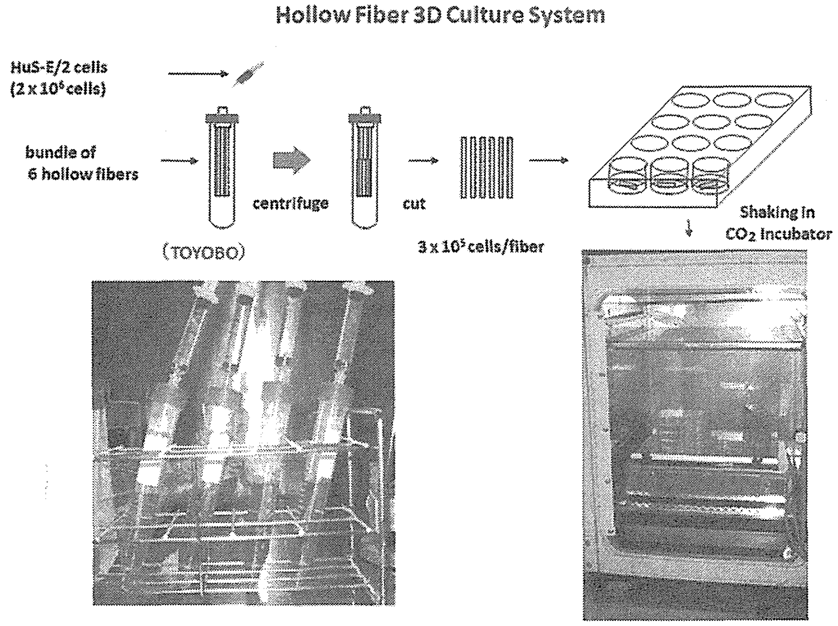

Fig. 4. 3-D hollow fiber culture. HuS-E/2 suspension was injected into the lumen of the hollow fiber system (HF; Toyobo Co., Osaka, Japan). The bundles were centrifuged to induce organoid formation. The lower 1.5 cm containing the organoid formation was then cut and cultured in 12-well plates (two capillary bundles per well) with gentle rotation using serum-free medium (Toyobo Co.) in a CO_2 incubator at 37°C. The number of cells was adjusted to 3×10^5 cells per two-capillary bundle at the start of each experiment.

genome and/or selection of clones during prolonged culture improved the productivity of infectious particles. This lack of production of infectious particles soon after infection may serve to avoid an early strong response from the host immune system, and demonstrates a novel mechanism of latent infection by HCV. Similarly, fluctuation in HCV proliferation was observed during the prolonged culture of 3-D-HuS-E/2 cells infected with bbHCV (54); this fluctuation was associated with a change in viral quasispecies, suggesting that an HCV strain having a growth advantage proliferates selectively and dominantly in these culture conditions. Because the progressive emergence of each dominant strain was only temporary, it is highly likely that the infection and proliferation of such an HCV strain is suppressed by cellular mechanism(s). Our results showed two cellular mechanisms functioning to do this. The first is the involvement of the innate immune system, as evidenced by the secretion of IFN-α during the first week of infection. The second mechanism is HCV-induced apoptosis. Although HCV-induced apoptosis was not found when HCV-1b was used for infection, it was found in all cases where HCV-2a was used, suggesting a higher cytopathic tendency of the HCV-2a genotype.

Mouse cells permissible to HCV infection

The development of prophylaxis and novel therapeutics to treat HCV infection has been hampered by the lack of suitable animal models, a deficit resulting from the limited species tropism of HCV. Chimpanzees are the only available immunocompetent *in vivo* experimental system, but their use is limited by ethical concerns, restricted availability and prohibitively high costs (59).

A convenient small-animal model supporting the HCV life cycle could significantly accelerate the preclinical testing of vaccine and drug candidates, as well as facilitate *in vivo* studies of HCV pathogenesis. A murine model was described in which overexpression of a uPA transgene resulted not only in neonatal bleeding disorders, but also in severe liver toxicity (60). Importantly, the diseased liver could be replaced by donor hepatocytes of murine origin, as well as by hepatocytes from rats, woodchucks, and humans once the uPA transgenic mice were backcrossed on an immunodeficient background. Mice with chimeric human livers that were inoculated with serum from HCV-positive donors developed prolonged HCV infections with high viral titers and evidence for active replication of the virus in chimeric human livers (61). At present, the chimeric human liver uPA/SCID mouse model is physiologically closest to a natural human infection and therefore represents the most successful small-animal model for HCV infection. Several shortcomings, however, limit its widespread use and application. Most importantly, the immunodeficiency required to allow successful xenotransplantation precludes studies on the adaptive immune response, immunopathology, and active immunization strategies (vaccine development). Second, only a few laboratories have reported successful generation of these chimeras, because this model requires high-quality human donor hepatocytes and the actual transplantation is difficult to carry out in small animals with a tendency to bleed. Finally, the efficacy of human hepatocyte engraftment is highly variable

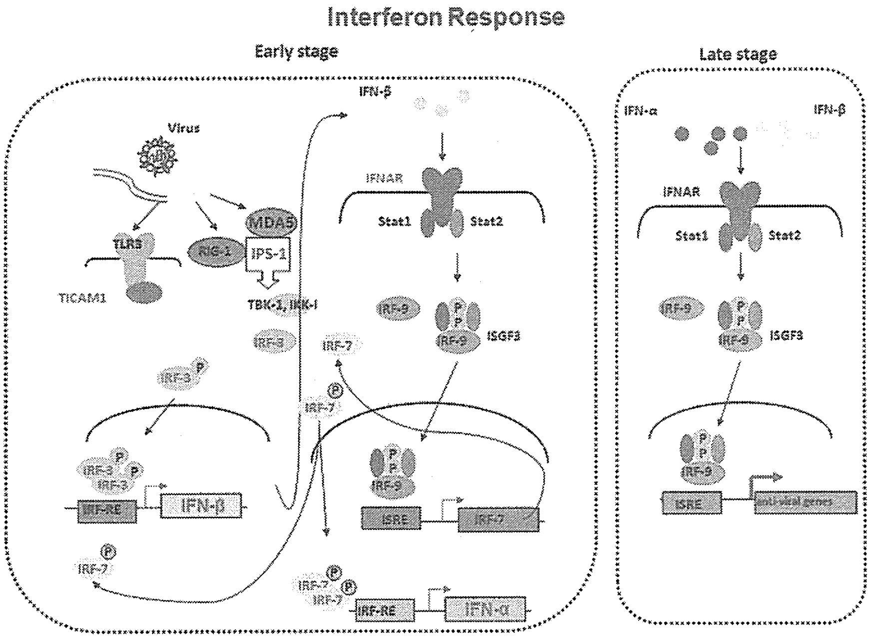

Fig. 5. Induction of interferon response by viral RNA. The cell detects viral RNA through the endosomal RNA sensor TLR3, and the cytoplasmic RNA sensors RIG-I and MDA5. Both pathways will lead to the activation of TBK-1 and IKK-I kinases, through the TICAM-1 adaptor molecule in the case of TLR3, or IPS-1 in the case of RIG-I and MDA5. These kinases will induce phosphorilization of interferon regulatory factor (IRF)-3, which will then dimerize and translocate to the nucleus. IRF-3 will then bind to the IRF response elements (IRF-RE) of IFN-β and lead to the induction of IFN-β expression. The IFN-β that is produced and secreted binds to the IFN receptor in an autocrine or paracrine manner to direct Janus Kinase Signal Transducer and Activator of Transcription (JAK-STAT) signaling and the interferon-stimulated gene factor 3 (ISGF3)-dependent expression of IRF-7 and other interferon-stimulated genes (ISG). IRF-7 will be phosphorylated by the activated TBK-1 and IKKε kinases, and form homo, or hetero-dimers with IRF-3, leading to further induction of IFN-β and -α genes. This signaling serves to amplify the IFN response by increasing the expression of IFN-β, IFN-α subtypes and ISG in a positive feedback loop.

in these animals, ranging from approximately 2% to 92% after additional treatment with an antibody to asialo- GM-1 (62).

The successful establishment of the HCV life cycle in mouse hepatocytes is another tempting alternative to overcome these problems. In addition to missing or incompatible positive regulators of HCV replication, dominant-negative restriction factors might be present in mouse hepatocytes. Altered or exacerbated innate antiviral responses, the inability of HCV proteins to overcome murine defenses, or mouse-specific restriction factors similar to those that control retroviral infection, such as Fv1, TRIM5α or APOBEC3 cytidine deaminases, could impair HCV replication in mouse cells.

In mammalian cells, the host detects and responds to infection by RNA-viruses, including HCV, by primarily recognizing viral RNA through several distinct pathogen recognition receptors (PRR), including the cell surface and endosomal RNA sensors TLR3 and TLR7, and the cytoplasmic RNA sensors RIG-I and MDA5 (Fig. 5) (63). The detection of virus infection by these receptors leads to the induction of IFN and their downstream IFN-inducible anti-viral genes through distinct signaling pathways (64). Type I IFN is an important regulator of viral infections in the innate immune system (65). Another type of IFN, IFN-lambda, affects the prognosis of HCV infection, and its response to antiviral therapy (66,67). Variations in the type or intensity of the antiviral response between hosts are known to restrict the tropism of certain viruses, such as myxoma virus, which is only permissive in mouse cells that have impaired IFN responses. Similarly, we previously reported that the impairment of IRF-7, and suppression of the interferon response improved HCV replication in immortalized primary human hepatocytes. (35)

Mutations impairing the function of the RIG-I gene and the induction of IFN were essential in establishing HCV infectivity in human HuH-7.5 cells (68). Similarly, the HCV-NS3/4a protease is known to cleave the IPS-1 adaptor molecule, inducing further downstream blocking of the IFN-inducing signaling pathway (69). These data clearly demonstrate that the host RIG-I pathway is crucial for suppressing HCV proliferation in human hepatocytes. Using a similar strategy, we investigated whether suppressing the antiviral host innate immune system conferred any advantage on HCV proliferation in mouse hepatocytes (70). We examined the possibility of HCV replication

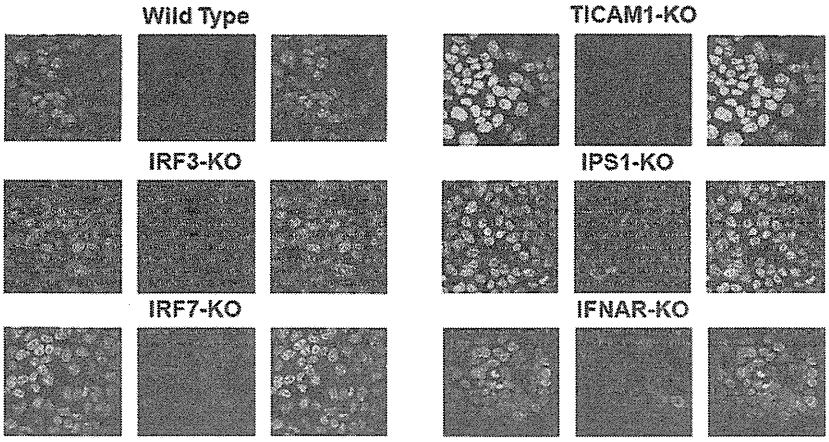

Fig. 6. Establishment of mouse hepatocyte lines permissive to J6/JFH1. Immunofluorescence detection of J6JFH1 proteins' expression 5 days after transfection of J6JFH1-RNA through electroporation into wild-type, IRF-3-ko, IRF-7-ko, TICAM1-ko, IPS-1-ko, and IFNAR-ko, freshly isolated primary hepatocytes. A highly sensitive polyclonal antibody extracted from HCV-patient serum (AbS3) was used for the detection.

in mice lacking the expression of key factors that modulate the type I IFN-inducing pathways (Fig. 6). Only gene silencing of IFNAR or IPS-1 was sufficient to establish spontaneous HCV replication in mouse hepatocytes.

To establish a cell line permissive for HCV replication, which is required for further *in vitro* studies of the HCV life cycle in mouse hepatocytes, we immortalized IFNAR- and IPS-1-ko mice hepatocytes with SV40 T antigen. Upon expression of the human (h)CD81 gene, these newly established cell lines were able to support HCV infection and replication for the first time in mouse hepatocytes. Using these cell lines, we demonstrated that the suppression of IPS-1 enhances HCV infection and replication in mouse hepatocytes through the suppression of both IFN induction and an IFN-independent J6JFH1-induced cytopathic effect. We also showed for the first time the importance of the HCV structural region for viral replication, as JFH1 chimera containing the J6 structure region showed a privilege for spontaneous replication over full-length JFH1 or the subgenomic JFH1 replicon. IRF-3-ko MEF were previously shown to support HCV replication more efficiently than wild MEF (71). As the knockout of IPS-1 mainly suppresses signaling in response to virus RNA detection, and maintains an intact IFN response and induction to other stimulants, it may result in minimum interference to adaptive immune responses as compared to IRF-3 or IFNAR-ko.

Conclusion

We have established an *in vitro* culture system that can support the entire life cycle of a variety of HCV isolates and genotypes. Although this *in vitro* model system may not completely reproduce the *in vivo* situation, we believe it is the first *in vitro* system showing HCV strain-dependent virus/cell interaction including induction of cellular apoptosis and/or evasion from the cellular innate immune response, which may make it a good tool for the analysis of virus/host interaction, together with the development of new anti-HCV strategies for the different bbHCV strains. We have also established hepatocyte lines from IPS-1-ko mice that support HCV replication and infection. These cell lines will be very useful in identifying other species' restriction factors and viral determinants required for the further establishment of a robust and efficient HCV life cycle in mouse hepatocytes. Further development of hCD81-transgenic IPS-1-ko mice may serve as a good model for the study of immunological responses against HCV infection. This mouse model can be used as a backbone for any further future models supporting robust HCV infectivity for the study of HCV pathogenesis, propagation and vaccine development.

ACKNOWLEDGMENT

The author wish to thank Dr. Jin Takahashi from the Japanese NIH, NIID for his help designing figure 5 in this paper.

DISCLOSURE

The authors declare no financial or commercial conflict of interest.

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Dendritic Cells from Oral Cavity Induce Foxp3$^+$ Regulatory T Cells upon Antigen Stimulation

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Abstract

Evidence is accumulating that dendritic cells (DCs) from the intestines have the capacity to induce Foxp3$^+$CD4$^+$ regulatory T cells (T-regs) and regulate immunity versus tolerance in the intestines. However, the contribution of DCs to controlling immunity versus tolerance in the oral cavity has not been addressed. Here, we report that DCs from the oral cavity induce Foxp3$^+$ T-regs as well as DCs from intestine. We found that oral-cavity-draining cervical lymph nodes contained higher frequencies of Foxp3$^+$ T-regs and ROR-γt$^+$ CD4$^+$T cells than other lymph nodes. The high frequency of Foxp3$^+$ T-regs in the oral-cavity-draining cervical lymph nodes was not dependent on the Toll like receptor (TLR) adaptor molecules, Myd88 and TICAM-1 (TRIF). In contrast, the high frequency of ROR-γt$^+$ CD4$^+$T cells relies on Myd88 and TICAM-1. *In vitro* data showed that CD11c$^+$ DCs from oral-cavity-draining cervical lymph nodes have the capacity to induce Foxp3$^+$ T-regs in the presence of antigen. These data suggest that, as well as in the intestinal environment, antigen-presenting DCs may play a vital role in maintaining tolerance by inducing Foxp3$^+$ T-regs in the oral cavity.

Citation: Yamazaki S, Maruyama A, Okada K, Matsumoto M, Morita A, et al. (2012) Dendritic Cells from Oral Cavity Induce Foxp3$^+$ Regulatory T Cells upon Antigen Stimulation. PLoS ONE 7(12): e51665. doi:10.1371/journal.pone.0051665

Editor: Gourapura J Renukaradhya, The Ohio State University, United States of America

Received August 22, 2012; **Accepted** November 6, 2012; **Published** December 18, 2012

Funding: This work was funded by a Grant-in-Aid for challenging Exploratory Research (SY) and a Grant-in-Aid for Scientific Research B (SY) from Japan Society for the Promotion of Science, the Mochida Memorial Foundation for Medical and Pharmaceutical Research (SY) and Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan. Specified financial supports by a MEXT Grant-in-Project 'the Carcinogenic Spiral' and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) are gratefully acknowledged. These funding bodies had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells (T-regs), constitute about 5–10% of peripheral CD4$^+$T cells and control immunological self-tolerance in rodents and human [1,2,3,4]. The expansion and induction of CD25$^+$Foxp3$^+$ T-regs in the periphery are controlled by professional antigen-presenting cells, dendritic cells (DCs) [5,6]. DCs can expand thymic-derived natural occurring T-regs [7,8,9]. DCs are the most efficient antigen presenting cells to induce Foxp3$^+$T-regs from Foxp3$^-$ precursors in the periphery [10,11]. Peripheral DCs directly control the numbers and homeostasis of Foxp3$^+$T-regs *in vivo* [12].

Foxp3$^+$T-regs induced by DCs in the intestine control the balance between inflammation and tolerance in the gut [13,14,15]. CD103$^+$DCs in the intestine use the retinoic acid-metabolizing enzyme retinaldehyde dehydrogenase and induce Foxp3$^+$T-regs to maintain oral tolerance [16,17]. Intestinal DCs use signaling through ß-catenin to induce Foxp3$^+$T-regs, which suppress Th17 and Th1 responses in the intestine [18]. Specific pathogens or Toll-like receptor (TLR) signals have been shown to induce Foxp3$^+$T-regs in the intestine [19,20]. Moreover, Foxp3$^+$T-regs control Th17 cells using interleukin (IL)-10 in the intestine [21,22]. Thus, Foxp3$^+$T-regs in the intestine are important in maintaining mucosal tolerance where there are vast numbers of commensal microbes and food antigens.

As in the intestine, many commensal microbes and food antigens also exist in the oral cavity [23,24,25,26]. Oral cavity is often involved with systemic immunological diseases such as graft versus host diseases, Stevens-Johnson syndrome, Behçet diseases, pemphigus vulgaris and Sjögren's syndrome. In addition, oral cavity is the place where many viruses, including influenza, herpes, common cold etc., start to infect. Therefore, it is important to identify how immune response is regulated in the oral cavity. Here we found that the DCs from oral cavity have the capacity to induce Foxp3$^+$T-regs. To our knowledge, this is the first report showing that DCs from the oral cavity induce Foxp3$^+$T-regs to maintain tolerance.

Results

The Frequency of Foxp3$^+$T-regs is Increased in Cervical Lymph Nodes (CLNs) in a Myd88/TICAM-1- Independent Manner

We considered whether Foxp3$^+$T-regs played an important role in the skin or oral cavity because the skin and oral cavity are exposed to many commensal microbes and antigens, like the intestine. First, we investigated the frequencies of Foxp3$^+$T-regs in lymph nodes (LNs) at different anatomical locations, which included skin- and oral-cavity-draining LNs. We found that cervical LNs (CLNs) contained a higher frequency of Foxp3$^+$ T-regs than other skin-draining LNs, such as axillary LNs (ALNs) and inguinal LNs (ILNs; paired t-test: $p<0.005$; Fig.1A arrow, Fig.1B and Fig.S1). CLNs contained a slightly, but significantly, higher frequency of Foxp3$^+$ T-regs than mesenteric LNs (MLNs; paired t-test $p<0.05$; Fig.1B). In MLNs, Foxp3$^+$T-regs are actively induced by CD103$^+$ DCs [16,17]. These data suggest that Foxp3$^+$T-regs may be also induced in CLNs, as in MLNs.

To investigate if the frequency of DCs correlates with the frequency of Foxp3$^+$T-regs, the frequency of CD11c$^+$DCs in total cells was compared between CLNs and ALNs (Fig.S2). The frequency of DCs was similar between CLNs and ALNs.

Recent reports showed that signals though TLR-2 induce Foxp3$^+$T-regs [20,27,28]. To examine whether signals from TLRs are required for the high frequency of Foxp3$^+$ T-regs in CLNs, we took advantage of Myd88 and TICAM-1 (TRIF) double knockout mice (Myd88/TICAM1 DKO), which lack all TLR signaling [29,30]. In Myd88/TICAM1 DKO mice, CLNs still contained a significantly higher frequency of Foxp3$^+$T-regs than inguinal LNs (ILNs; paired t-test: $p<0.05$; Fig.1B). The frequency of Foxp3$^+$T-regs in CLNs did not differ between Myd88/TICAM1 DKO and wild-type (WT) mice (t-test: $p=0.09$; Fig.1B, 1C). The frequency of Foxp3$^+$T-regs in MLNs did not differ between Myd88/TICAM1 DKO and WT mice also (t-test: $p=0.4$; Fig.1B).

Thus, Foxp3$^+$T-regs are increased in CLNs in a Myd88/TICAM1-independent manner, suggesting that TLR signals are not involved in the increase in Foxp3$^+$ T-regs in CLNs.

The Frequency of ROR-γt$^+$ CD4$^+$T Cells is Increased in CLNs in a Myd88/TICAM1- Dependent Manner

The induction of Foxp3$^+$T-regs in the intestine is reciprocally controlled by Th17 [18,31]. To examine the balance between Th17 and Foxp3$^+$T-regs, we next compared the frequencies of ROR-γt$^+$ CD4$^+$T cells in different anatomical locations. ROR-γt is a transcription factor expressed by Th17 cells [32]. We found that CLNs had a significantly higher frequency of ROR-γt$^+$ CD4$^+$T cells than other skin-draining LNs and spleen (paired t-test: $p<0.05$; Fig.2A closed arrows and Fig.2B). As expected, MLNs contained a higher frequency of ROR-γt$^+$ CD4$^+$T cells than other LNs (Fig.2A gray arrows and Fig.2B).

To assess whether signals from microbes through TLRs are required for the induction of ROR-γt$^+$ CD4$^+$T cells in CLNs, we investigated Myd88/TICAM1 DKO mice. In Myd88/TICAM1 DKO mice, frequencies of ROR-γt$^+$ CD4$^+$T cells did not differ between CLNs and ILNs (paired t-test: $p=0.05$; Fig.2B). Moreover, the frequency of ROR-γt$^+$ CD4$^+$T cells in CLNs was significantly reduced in Myd88/TICAM-1 DKO mice (t-test: $p<0.05$; Fig.2B). The frequency of ROR-γt$^+$ CD4$^+$T cells in MLNs did not differ between Myd88/TICAM1 DKO and WT mice also (t-test: $p=0.1$; Fig.2B).

Thus, both Foxp3$^+$T-regs and Th17 may be induced in CLNs. However, Myd88/TICAM1 signaling is important for the development of ROR-γt$^+$CD4$^+$-Th17 T cells in CLNs (Fig.2B), but not for the induction of Foxp3$^+$T-regs (Fig.1B).

CLNs are Oral-cavity-draining Lymph Nodes

We considered that the higher frequency of Foxp3$^+$T-regs in CLNs may reflect their response to antigens in the oral cavity. To confirm whether CLNs were draining LNs from the oral cavity, we investigated the proliferation of transferred OT-II CD4$^+$-T cells in CLNs after sublingual (s.l.) administration of ovalbumin (OVA; Fig.3). OT-II mice are commonly used OVA-specific CD4$^+$ T-cell receptor transgenic mice [7,10,28]. In the absence of OVA s.l. administration, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-II T cells did not divide, in CLNs or ALNs (Fig.3, top). With OVA s.l. administration, CFSE-labeled OT II T cells divided well in CLNs (Fig.3, bottom arrow), but not in ALNs. Thus, we confirmed that CLNs were draining LNs of the oral cavity because s.l.-administered OVA antigen was presented to OT II CD4$^+$-T cells.

DCs from Oral-cavity-draining CLNs Locate Close to Foxp3$^+$T-regs and have the Capacity to Induce Foxp3$^+$ T-regs on Antigen Stimulation

Next, to investigate the interaction between DCs and Foxp3$^+$T-regs in CLNs, we microscopically examined CLNs. We found that Foxp3$^+$T-regs and CD11c$^+$ DCs were closely located, as reported previously in MLNs [33] (Fig.4A). This suggests that DCs from CLNs may induce Foxp3$^+$T-regs as DCs do in MLNs.

To determine whether DCs from the oral cavity can in fact induce Foxp3$^+$T-regs, we compared the capacity to induce Foxp3$^+$T-regs *in vitro* using DCs from ALNs, MLNs, and oral-cavity-draining CLNs. Purified CD11c$^+$ DCs from CLNs, ALNs, or MLNs were cultured with OT II CD4$^+$T cells with or without antigen for 5 days. In the presence of antigen, CLN DCs induced a higher frequency of Foxp3$^+$T-regs compared with ALN DCs (paired t-test: $p<0.005$; Fig.4B, 4C). The frequency of Foxp3$^+$T-regs induced by antigen plus DCs did not differ between the culture with CLN DCs and that with MLN DCs (paired t-test: $p=0.878$; Fig.4C).

These results indicated that DCs from the oral-cavity-draining CLNs had the capacity to induce Foxp3$^+$T-regs with antigen, as DCs from MLNs do.

CD103$^+$DCs may not be Involved in Inducing Foxp3$^+$ T-regs in Oral-cavity-draining CLNs

To determine whether DCs from the oral cavity contain a specific DC subset to induce Foxp3$^+$T-regs as in the intestine, we first performed real-time PCR. When we investigated the mRNA expression of retinal dehydrogenase 2 (RALDH2), transforming growth factor (TGF)-ß, and IL-10, there was no difference between DCs from CLNs and ALNs (Fig.4A). DCs from MLNs had higher mRNA expression of RALDH2 as previously reported (Fig.5A). We also measured the protein production of TGF-ß1 and IL-10 in the culture supernatant. TGF-ß1 was not detected in the culture supernatants of CLN DCs with or without latent TGF-ß activation (data not shown). We did not detect IL-10 in the culture supernatants from CLN DCs and OT II CD4$^+$T cells without peptide in Fig.4B and 4C (data not shown). Theses results indicate that TGF-ß1, IL-10 and RALDH2 may not involve in the induction of Foxp3$^+$T-regs by CLN DCs.

To investigate whether CD103$^+$DCs play a role in inducing Foxp3$^+$T-regs in CLNs, we compared the frequency of CD103$^+$DCs in each location. However, oral cavity-draining CLNs had a lower frequency of CD103$^+$ DCs than MLNs (Fig.5B).

Plasmacytoid DCs have a capacity to induce Foxp3$^+$T-regs [34,35,36,37]. Epidermal Langerhans cells and migratory dermal DCs have also been reported to induce Foxp3$^+$T-regs [38,39,40]. However, the frequencies of plasmacytoid DCs and migratory class II^{high} DCs did not differ between CLNs and ALNs (Fig.5C). Next, we investigated the classical CD8$^+$ and CD8$^-$ DC subsets in CLNs and ALNs. The frequency of CD8$^+$ DCs was similar between CLNs and ALNs (paired t test: $p=0.065$) (Fig.5D). However, CLNs had a significantly higher frequency of CD8$^-$ DCs than ALNs (paired t test: $p<0.0005$) (Fig.5D).

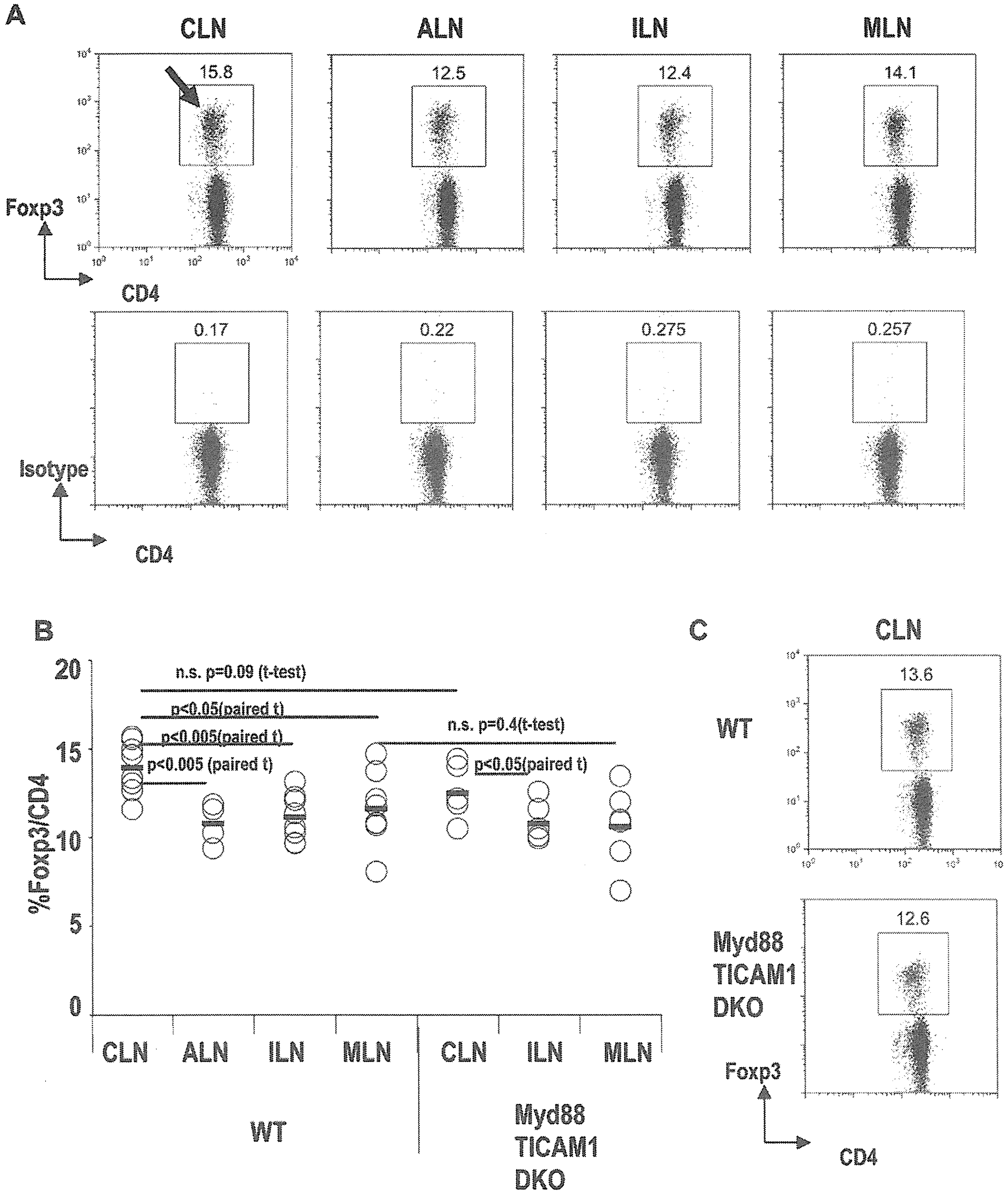

Figure 1. Frequency of Foxp3$^+$ T-regs in cervical lymph node is increased in a Myd88/TICAM-1 independent manner. (A) Cervical lymph nodes (CLN), axillary lymph nodes (ALN), inguinal lymph nodes (ILN), and mesenteric lymph nodes (MLN) from wild type B6 mice were analyzed for the expression of Foxp3. The isotype control is shown at the bottom. Plots were gated on CD4$^+$ T cells. Representative of seven separate experiments. (B) As in (A), but CLN, ALN, ILN and MLN from wild-type B6 mice (WT) or Myd88/TICAM1 double knockout mice (Myd88/TICAM1 DKO) were analyzed for the expression of Foxp3. Summary from seven separate experiments. P value provided is by paired t-test or t-test. "n.s." = "not significant". (C) As in (B), but a representative of CLN is shown.
doi:10.1371/journal.pone.0051665.g001

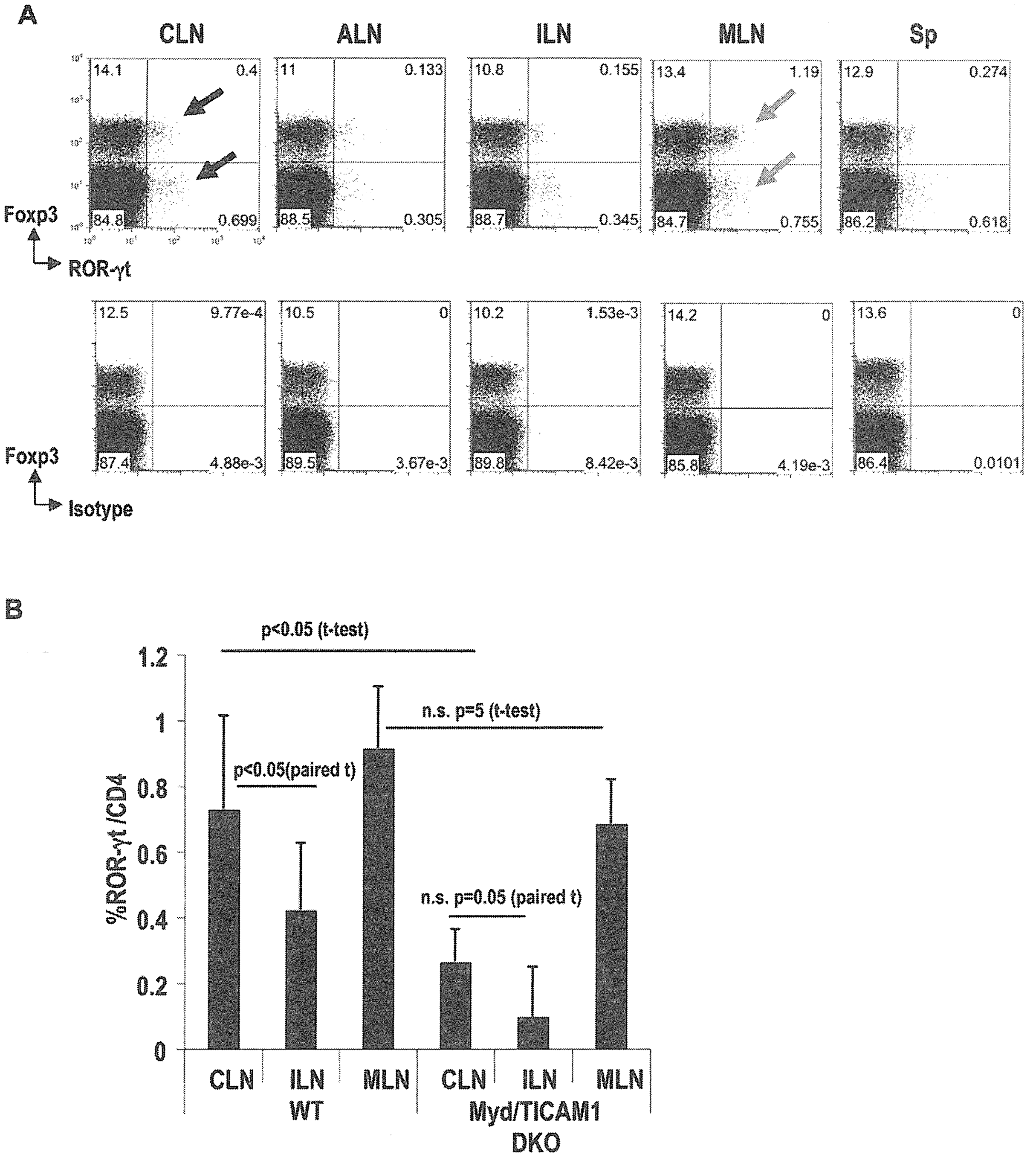

Figure 2. Frequency of ROR-γt^{+}CD4^{+} T cell in cervical lymph node is increased in a Myd88/TICAM-1 dependent manner. (A) CLN, ALN, ILN, MLN, and spleen (Sp) from WT B6 mice were analyzed for the expression of Foxp3 and ROR-γt. The plots were gated on CD4^{+} T cells. Isotype staining for ROR-γt is shown at the bottom. Representative of three separate experiments. (B) As in (A), but cells from WT mice or Myd88/TICAM-1 DKO mice were analyzed for the expression of ROR-γt and CD4. The graphic shows a summary from two separate experiments. P value provided is by paired-t test or t-test. "n.s." = "not significant".
doi:10.1371/journal.pone.0051665.g002

These results suggest that CD103^{+} DCs and retinoic acid may not contribute to inducing Foxp3^{+}T-regs in CLNs. It is possible that the classical CD8^{-} DC subset in CLNs may participate in the induction of Foxp3^{+}T-regs. Further studies are required.

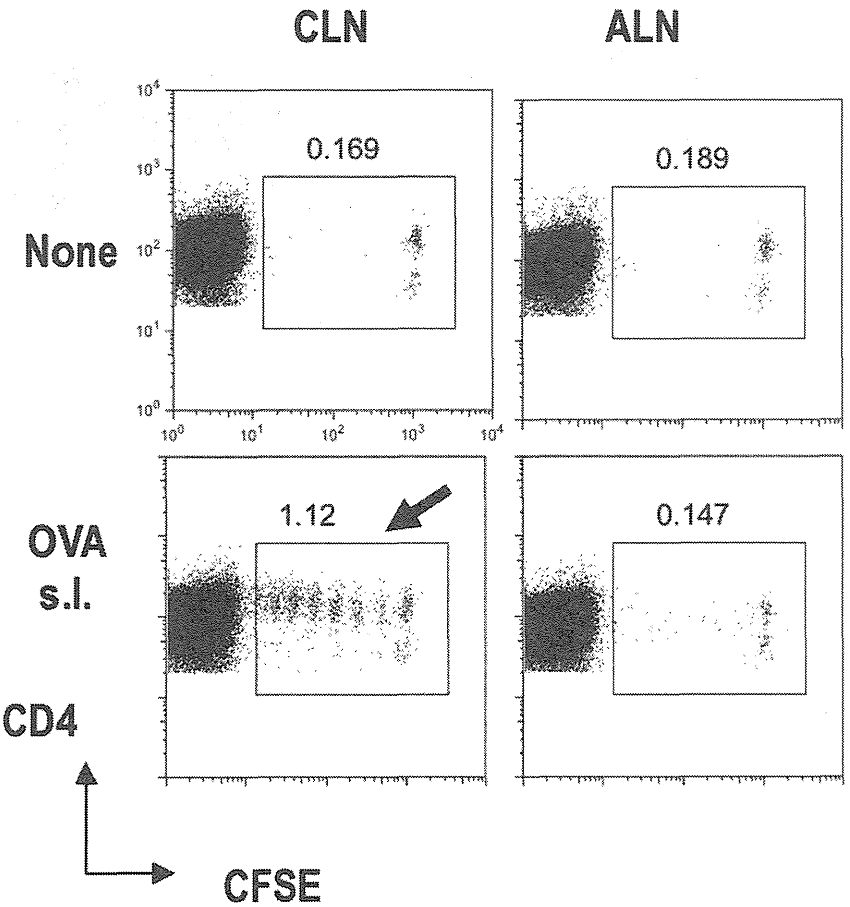

Figure 3. Cervical lymph nodes are draining lymph nodes from the oral cavity. CFSE-labeled OTII CD4$^+$T cells were adoptively transferred into B6 mice on day −1. On day 0, 500 µg of OVA was administered sublingually (s.l.). CLN or ALN was analyzed for CFSE dilution at day 3. One of two similar experiments is shown for the FACS plots. Plots were gated on CD4$^+$T cells.
doi:10.1371/journal.pone.0051665.g003

Discussion

The oral cavity is exposed to many antigens and commensal organisms every day [23,24,25,26]. The oral cavity is frequently associated with systemic immunological disorders, such as graft versus host diseases, Stevens-Johnson syndrome and Behçet diseases. However, it is unknown how tolerance in the oral cavity is maintained. Here, we showed that the balance between Th17 and Foxp3$^+$T-regs may play a role in maintaining tolerance in the oral cavity. We found that the frequencies of Foxp3$^+$T-regs and ROR-γt $^+$CD4$^+$T cells were increased in oral-cavity-draining CLNs, compared with skin-draining LNs and mesenteric LNs. DCs from oral-cavity-draining CLNs have the capacity to induce Foxp3$^+$ T-regs *in vitro* on antigen stimulation, as much as DCs from mesenteric LNs do. These data suggest that the induced Foxp3$^+$T-regs in oral-cavity-draining CLNs may be important in maintaining mucosal tolerance in response to microbes and food antigens in the oral cavity.

Although some TLR signaling is involved in inducing Foxp3$^+$ T-regs [20,27,28,41,42], the high frequency of Foxp3$^+$ T-regs in CLNs was not dependent on Myd88/TICAM1 (Fig. 1). Thus, TLR signaling is apparently not involved in the induction of Foxp3$^+$T-regs in the oral cavity. However, we cannot exclude the possibility that some specific microbes may be involved in the inducing Foxp3$^+$T-regs in the oral cavity in a Myd88/TICAM1 independent manner. For example, Atarashi et al recently showed that *Clostridum* induces Foxp3$^+$T-regs in the colon in a Myd88-independent manner [19]. It might be interesting to investigate if there are specific microbes that contribute to the induction of Foxp3$^+$T-regs in the oral cavity, especially as the bacterial community varies between the oral cavity and gut [26].

Our results showed that the high frequency of ROR-γt$^+$CD4$^+$T cells was dependent on Myd88/TICAM1 in the oral-cavity-draining CLNs (Fig.2). This is consistent with recent findings in skin [43]. Th17 cells in skin are reduced in Myd88/TICAM1 knockout mice and skin-resident commensal bacteria induce Th17 cells in a Myd88- and IL-1 receptor-dependent manner [43]. It is also known that some microbes induce Th17 using TLR signals; for example, Th17 cells induced by *Chlamydia* infection are reduced in Myd88 KO mice [44]. Thus, it is possible that TLR signals through some oral microbes are responsible for the increase of ROR-γt $^+$CD4$^+$T cells in oral-cavity-draining CLNs. Interestingly, the intestine may use a different mechanism from the oral cavity and skin to maintain Th17 cells, because Th17 cells are not reduced in the intestine in Myd88/TICAM1(TRIF)-knock out mice [45,46].