Chapter-7

Discussion
Infection with the hepatitis C virus (HCV) leads to chronic hepatitis C (CHC) in 50% to 80% of individuals. The recognition of HCV by the host, triggers pathways that lead to type I interferon (IFN-α and IFN-β) production, and to the induction of an antiviral state (Bigger et al., 2004; Sarasin et al., 2008).

During TLR signaling, TLRs bind to their ligand and then interact with the adaptor protein called myeloid differentiation primary response gene 88 (MyD88) (Kawai et al., 2007). MyD88 then recruits members of the IL-1 receptor-associated kinase (IRAK) family such as IRAK1 and IRAK4, which activate TNF receptor-associated factor 6 (TRAF6). TRAF6 is an E3 ubiquitin ligase that catalyzes the formation of Lys-63-linked polyubiquitination on TRAF6 itself and IκB kinase c (IKKe also known as NEMO) (Deng et al., 2000; Lamothe et al., 2007). Subsequently, a complex of TAK1, TAK1 binding protein 2 (TAB2), and TAB3 is recruited to TRAF6 (Wang et al., 2001; Kanayama et al., 2004). TAK1 activates the IKK complex, leading to NF-kB activation and induction of proinflammatory cytokine expression, which is also enhanced by TRAF6 and MyD88-activated IRF5 (Takaoka et al., 2005). In addition, upon viral infection, TRAF6 forms a complex with IRF7 together with TRIF (Gohda et al., 2003), MyD88 (Kawai et al., 2004; Honda et al., 2004), IRAK4, and IRAK1 (Uematsu et al., 2005). IRF7 is then phosphorylated by IRAK1 and/or IKKa (Hoshino et al., 2006), which result in dimer formation and nuclear translocation of IRF7, leading to the production of type I IFNs. Thus, TRAF6 plays a pivotal role in TLR signaling.
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RLHs, such as RIG-I and the protein product of the melanoma differentiation-associated gene 5 (MDA5), contain two functional domains: an RNA helicase domain and a caspase recruitment domain (CARD) (Yoneyama et al., 2005). The RNA helicase domain recognizes viral RNA, synthetic double-stranded RNA (dsRNA), and 5’-triphosphate RNA (Pichlmair et al., 2006), and the CARD domain interacts with the CARD-like domain of the IFN-β promoter stimulator-1 (IPS-1, also known as MAVS/VISA/Cardif). Upon viral infection, IPS-1 associates with RIG-I or MDA5 at the mitochondrial outer membrane via the CARD-CARD interaction, which is essential for triggering downstream signaling that activates NF-kB and IRF. RLH signaling has been proposed to bifurcate at IPS-1 into the TRAF3-dependent IRF activation pathway and the TRAF6-dependent NF-kB activation pathway (Bowie et al., 2008). Sequence analysis of MAVS based upon the Ye et al., 2002 indicates that it contains two consensus TRAF6 binding motifs, 153-PGENSE-158 and 455-PENENY-460. Mutation of TRAF6 binding sites in IPS-1 resulted in a marked reduction of IPS-1-induced NF-kB activation in a transient transfection assay (Xu et al., 2005). Furthermore, the role of TRAF6 in IRF activation during RLH signaling has never been adequately addressed.

Konno et al., (2009) clearly demonstrate that TRAF6 plays a critical role in RLH signaling. The absence of TRAF6 resulted in enhanced viral replication and a significant reduction in the production of IL-6 and type I IFNs after infection with RNA virus. Activation of NF-kB and IRF7 was significantly impaired during RLH signaling in the absence of TRAF6. TGFb-activated kinase 1 (TAK1) and MEKK3, whose activation by TRAF6 during TLR signaling is involved in NF-kB activation, were not essential for RLH-mediated NF-kB activation. TRAF6-deficiency impaired
cytosolic induced antiviral responses, and this impairment was due to defective activation of NF-kB and IRF7.

Jiang et al, (2004) found that TLR3 recruits TRAF6 via adapter TRIF through a TRAF6-binding sequence in TRIF (Amino acids 250 – PEEMSW – 255). Mutation of this TRAF6-binding sequence abolished the interaction of TRIF with TRAF6 but not with TLR3. Interestingly, mutation of the TRAF6-binding site of TRIF only abolished its ability to activate NF-kB but not IRF3, suggesting that TLR3-mediated activation of NF-kB and IRF3 bifurcate at TRIF.

Hepatitis C virus (HCV) frequently associated with hepatocellular carcinoma (HCC) and B cell lymphoma. Machida et al., (2004) demonstrated that acute and chronic HCV infection caused a 5 to 10 fold increase in mutation frequency in Ig heavy chain, BCL-6, p53, and β-catenin genes of in vitro HCV-infected B cell lines and HCV-associated peripheral blood mononuclear cells, lymphomas, and HCCs. The nucleotide-substitution pattern of p53 and β-catenin was different from that of Ig heavy chain in HCV infected cells, suggesting two different mechanisms of mutation. In addition, the mutated protooncogenes were amplified in HCV-associated lymphomas and HCCs, but not in lymphomas of nonviral origin or HBV-associated HCC. HCV induced error-prone DNA polymerase ζ, polymerase ι and activation-induced cytidine deaminase, which together, contributed to the enhancement of mutation frequency, as demonstrated by the RNA interference experiments. These results indicate that HCV induces a mutator phenotype and may transform cells by a hit-and-run mechanism. This provides a mechanism for HCV virus induced mutagenesis in the host.
Based upon previous literatures the possible existence of sequence variations in the TRAF6 interacting motifs of MAVS and TRIF were analyzed for the first time in the current study. The sequences which code for the TRAF6 binding motifs of MAVS and TRIF were completely matching with the reference sequences, and considered as highly conserved in all the study population (See Fig. 6.3 and 6.4). Thus our study concludes that the abruption of TRAF6 interactions with MAVS and TRIF were not associated with the sequence variations.

Viral persistence is a hallmark of HCV, wherein continual virus replication underlies the pathogenesis and liver disease associated with chronic infection. Our results provide evidence that links HCV persistence with viral control of the host response to infection. We found that HCV infection signals the activation of IFN-β and host response in TLR and RIG-I-dependent pathways and that TRIF and MAVS are essential components of these signals that targeted and cleaved during HCV infection in vivo (See Fig 6.6 and Table 6.2). Similar observation made by Ballecave et al., 2010, concluding that cleavage of MAVS is specific for hepatitis C, because it was never detected in patients with other chronic liver diseases, including chronic hepatitis B.

These results confirm and extend the observations of others innate antiviral immuno activation of ISG, that have been reported during the experimental procedures (Meylan et al., 2005). Many viruses antagonize IRF-3 through varied mechanisms that support their replication (Sen GC, 2001), underscoring the central role of IRF-3 and IFN in antiviral immune defense (Sato et al., 2000).

Bellecave et al., (2010) demonstrated, MAVS is rapidly cleaved at C508 by NS3/4A during HCV infection, resulting in release of MAVS from the mitochondrial
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membrane and destabilization and loss of its interaction with RIG-I. Thus, mitochondrial membrane association appears essential for MAVS function. MAVS likely requires interaction with other mitochondria-associated factors that participate in protein recruitment and/or signaling of IFN activation. In support of this idea, recent studies suggest that MAVS may recruit a variety of effectors that confer signaling by RIG-I or the related helicase, MDA5, which may bind to distinct RNA pathogen associated molecular patterns to initiate the host response independently of RIG-I (Kawai et al., 2005). Importantly, NS3/4A blocks signaling by both RIG-I and MDA5 (Yoneyama et al., 2005), consistent with a central role for MAVS in these respective pathways.

Our results support the concept that control of the host response by HCV, in the presence of infection initially triggers IRF-3 activation through TRIF and MAVS dependent signaling during a low viral abundance (See Table 6.5, 6.7 and 6.9). However, as the viral load increases, the NS3/4A protease accumulates to a threshold level within the cell, ultimately resulting in cleavage of TRIF and MAVS. This process terminates IFN stimulation of the host, promoting viral persistence. Such a model predicts that IFN products would be continuously produced as a result of asynchronous viral spread within the liver during chronic infection, because the host response would be induced in newly infected cells until the point of TRIF and MAVS cleavage. This prediction is consistent with previous study showing expression of intrahepatic ISGs during chronic HCV infection (Smith et al., 2003) and our observations of varied MAVS and TRIF interactions with TRAF6, and cleavage of MAVS and TRIF leading to disruption of their interaction with TRAF6 as demonstrated in the liver tissue of chronic hepatitis C patient has been supported by
similar observations of other workers (Li et al., 2005a; Ferreon et al., 2005 and Li et al., 2005b) in cell culture experiments.

In the liver biopsy specimens of our HCV-infected patient cohorts, there was evidence to show that TRAF6 interaction with MAVS and TRIF corresponded with an abundance of IFN-β (see Fig. 6.13), whereas non-interacting TRAF6-MAVS and TRAF6-TRIF, with cleaved MAVS and TRIF was associated with a lack of IFN-β expression (see Fig. 6.6). Recent studies show that only a portion of total nonstructural proteins associate with the HCV replicase (Quinkert et al., 2005) as well as NS3/4A can independently localize to the mitochondria (Kasprzak et al., 2005), supporting roles for it both in viral replication and host defense regulation.

When the TRAF6 interactions with MAVS and TRIF were compared among patients with different HCV genotypes, it was observed that all genotype 1 patients were not showing TRAF6 interactions with either of MAVS and TRIF where as 15 out of 43 genotype 3 patients were showing MAVS and TRAF6 interactions, and 12 out of 43 genotype 3 patients have shown interactions between TRIF and TRAF6 proteins (see Table 6.4). IFN-β levels were at significantly low levels in genotype 1 patients compared to the genotype 3 patients, though the HCV viral load was not significantly different between genotype 1 and 3 of chronic hepatitis C patients (see Table 6.8). After therapy the viral load was found to be significantly higher in the Genotype 1 patients compared to genotype 3 patients, while IFN-β levels was higher in the treated genotype 3 patients compared to genotype 1 patient (see Table 6.17).

The observations reported by Bellecave et al., 2010, was different from our observations. They found easier-to-treat GTs 2 and 3 cleave MAVS more extensively than the difficult-to-treat GTs 1 and 4. Accordingly, MAVS cleavage was detected in
a larger proportion of patients infected with GTs 2 and 3 than with GTs 1 and 4 (56.6% versus 42.6%). Given the role of MAVS in IFN-β induction, Sarasin et al., 2008, reported a lower rate of ISG induction in pretreatment biopsy specimens of patients infected with GTs 2 and 3 when compared with GTs 1 and 4.

In the context of IFN therapy for HCV infection, TRIF and MAVS cleavage by NS3/4A would attenuate this loop and the expression of those ISGs normally amplified and/or induced through RIG-I and TLR-3-dependent processes. Such a mechanism could limit the efficacy of IFN-based therapies.

Our results in table 6.2 and 6.11 showing an increase in incidence of MAVS-TRAF6 interaction from 29% in treatment naïve to 60% in treated patient (non-responders) suggest that these therapies may have dual mechanisms, both suppressing HCV replication by ribavirin, while concomitantly releasing the host response from the HCV imposed blockade by NS3/4A cleavage of TRIF and MAVS, which increases the IFN-β level and diversity of ISG expression (Yoneyama et al., 2004).

We conclude that MAVS and TRIF are unable to interact with TRAF6, whenever they are targeted and cleaved by NS3/4A, which is consistent with the reports in the literature (Li et al., 2005a; Ferreon et al., 2005 and Li et al., 2005b). Disruption of TRAF6 interaction with MAVS and TRIF in HCV infection in vivo leads to low serum IFN-β levels (see table 6.7 and 6.16). HCV protease inhibitors are currently being developed for clinical applications (Tan et al., 2004). Targeting of the NS3/4A-MAVS or -TRIF interface by protease inhibitors may therefore enhance IFN actions. This therapeutic control of MAVS and TRIF may explain the remarkable effectiveness and rapidity with which such compounds have been shown to suppress
HCV replication and viremia in patients participating in early phase I clinical trials (Lamarre et al., 2003).