

FIGURE 7. Proposed model of TLR3 processing. (1) TLR3 is neo-synthesized and *N*-glycosylated in the ER. (2) Then, it crosses the Golgi apparatus where it is fully glycosylated to become EndoH resistant. TLR3 exits the Golgi to enter the endosome membrane (3) where it is cleaved by cathepsins (4). The two proteolytic fragments remain associated to fully signal (5).

ternal loop (residues 335–343) during mammals' evolution (30) suggests that cleavage is an important step in the biology of TLR3. Remarkably, our data confirm that the two proteolytic fragments of the ECD of TLR3 have prolonged half-lives (26) and demonstrate that they remain associated, suggesting that the noncovalent interactions between the adjacent LRRs known to stabilize the ECD of TLRs (36) have been preserved. Furthermore, the absence of detectable amounts of Golgi-modified TLR3_{FL+} in resting immune and nonimmune cells (Figs. 1A, 1D–F, 3B, 3C) indicates that cleaved/associated TLR3 is the almost exclusive form of the receptor present in endolysosomes, where the encounter with exogenous dsRNA is known to occur (37). The lack of appropriate ligand prevented us from visualizing directly TLR3 bound to dsRNA. However, the physical association of TRIF with TLR3_{C-ter}, but not TLR3_{FL}, after activation with Poly(I:C) in NCI-H292 cells (38), combined with the absence of free TLR3_{C-ter} in those cells, indicates that cleaved/associated TLR3 is the main form of the receptor recognizing Poly(I:C). The single cleavage, without further trimming, may explain why the two long-lived fragments of TLR3 remain associated to bind dsRNA. In contrast, although it was proposed that some TLR9 fragments could remain associated (8, 39), the C-terminal fragment of the receptor is viewed as the major form of the functional receptor, binding agonist CpG oligodesoxynucleotides with high affinity and being able to efficiently recruit the adaptor protein MyD88 (8, 10).

The streamlined transfer to endolysosomes, followed by rapid cleavage, explains why endogenous TLR3 fragments were abundant in resting cells of every type analyzed, whereas TLR3_{FL} was difficult to detect. In contrast, comparable amounts of TLR3_{FL} and TLR3 fragments were observed in HEK293 cells, suggesting an imbalance between the high expression of exogenous TLR3 and the availability of the chaperone protein Unc93b1 in those cells (26). Indeed, exogenous TLR3 was abundant in the ER, whereas endogenous TLR3 was found mostly in the endolysosomes. Moreover, the half-lives of the fragments from transfected TLR3 were shorter compared with endogenous TLR3 (compare Fig. 2B with Fig. 2A). These differences should be kept in mind when studying the biology of endosomal TLRs in HEK293 cells.

TLR3 cleavage could increase or decrease the sensitivity of the receptor and/or modify its specificity for different ligands. Our functional studies reveal that, in TLR3-transfected HEK293 cells, the cleavage increased the sensitivity to HMW and LMW Poly(I:C). The increased sensitivity of cleaved/associated TLR3 remains perplexing. Thus, cleavage could somehow increase the affinity of the ECD for its ligands or ease the conformational change that may occur in the presence of dsRNA (39) and that may facilitate the recruitment of TRIF. In agreement with Qi et al. (26), we observed that TLR3_{C-ter} by itself was consistently unable to trigger a strong response to dsRNA. A difference in timing (6 versus 18 h) might explain, in part, the variance between those results and recently published data that showed an equal response to Poly(I:C) with either TLR3-WT or TLR3_{C-ter} (22). Whatever the residual activity of TLR3_{C-ter}, its physiological importance is uncertain, because cleaved/associated TLR3 appears to be the predominant form of the endogenous receptor present in the endolysosomes where recognition of dsRNA takes place.

The central role of cleaved/associated TLR3 highlights the importance for dsRNA binding affinity and sensitive signaling of two distinct ligand-binding sites, each present on one proteolytic fragment. Moreover, the increased sensitivity to Poly(I:C) and the remarkable stability of this form of the receptor allows the reconciliation of some apparently discordant results from the literature. Indeed, one group reported the absence of inhibition of TNF production by RAW macrophages treated for 12 h with cathepsin inhibitors and then for 2 h with 100 μ g/ml of Poly(I:C) (8), whereas another group showed a strong suppression of TNF production by the same cells in response to 1 μ g/ml of Poly(I:C) (9). These different outcomes may be due to differences in the concentration of ligand used, with high concentrations of dsRNA being able to activate the less efficient TLR3_{FL} in these cells. In addition, our data show that 12 h of Z-FA-fmk pretreatment is not sufficient to suppress the expression of TLR3 fragments in NSCLC cells, suggesting that the lack of inhibition by Z-FA-fmk of cells activated with moderate concentrations of Poly(I:C) could have resulted from the persistence of some cleaved/associated TLR3 at the time of stimulation.

In conclusion, TLR3 provides the first example, to our knowledge, of endosomal receptor maturation by cleavage followed by conversion into a functional cleaved/associated form of the protein. Considering that cleavage of WT-TLR3 is necessary for signaling, cleaved/associated TLR3 is the principal (and possibly exclusive) signaling receptor, and noncleavable TLR3 is able to signal, an intriguing conclusion of the present work is that the licensing consequence of TLR3 cleavage for signaling is not the separation of the two fragments. Further studies are required to fully evaluate the structural and functional consequences of TLR3 processing in vitro and in vivo, as well as to determine to what extent some aspects of TLR3 biology might apply to the other endolysosomal TLRs.

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Disclosures

The authors have no financial conflicts of interest.

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TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis

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Keywords: interferon-inducing pathway, necroptosis, RIP signaling, TLR3, TICAM-1, TRIF

Abbreviations: CTL, cytotoxic T lymphocyte; DAI, DNA-dependent activator of IFN-regulatory factors; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group box 1; HSP, heat shock protein; mDC, myeloid dendritic cell; NK, natural killer; NLR, NOD-like receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; RIP, receptor-interacting protein kinase; TICAM-1, Toll-IL-1-homology domain-containing adaptor molecule 1; TLR, Toll-like receptor; TNF α , tumor necrosis factor α ; TNFR1, TNF α receptor 1

The engagement of Toll-like receptor 3 (TLR3) leads to the oligomerization of the adaptor TICAM-1 (TRIF), which can induce either of three acute cellular responses, namely, cell survival coupled to Type I interferon production, or cell death, via apoptosis or necrosis. The specific response elicited by TLR3 determines the fate of affected cells, although the switching mechanism between the two cell death pathways in TLR3-stimulated cells remains molecularly unknown. Tumor necrosis factor α (TNF α)-mediated cell death can proceed via apoptosis or via a non-apoptotic pathway, termed necroptosis or programmed necrosis, which have been described in detail. Interestingly, death domain-containing kinases called receptor-interacting protein kinases (RIPs) are involved in the signaling pathways leading to these two cell death pathways. Formation of the RIP1/RIP3 complex (called necrosome) in the absence of caspase 8 activity is crucial for the induction of necroptosis in response to TNF α signaling. On the other hand, RIP1 is known to interact with the C-terminal domain of TICAM-1 and to modulate TLR3 signaling. In macrophages and perhaps tumor cell lines, RIP1/RIP3-mediated necroptotic cell death can ensue the administration of the TLR agonist polyI:C. If this involved the TLR3/TICAM-1 pathway, the innate sensing of viral dsRNA would be linked to cytopathic effects and to persistent inflammation, in turn favoring the release of damage-associated molecular patterns (DAMPs) in the microenvironment. Here, we review accumulating evidence pointing to the involvement of the TLR3/TICAM-1 axis in tumor cell necroptosis and the subsequent release of DAMPs.

Introduction

Cell death is an important process for both development and homeostasis in multicellular organisms. The mode of cell death is closely associated with other biological responses occurring within the host, including inflammation. Cell death has been categorized as apoptotic or necrotic and, until recently, apoptosis

had been considered as a synonym of programmed cell death.¹ Caspases are a family of cysteine proteases that mediate apoptotic cell death in response to ligands of death receptors, including tumor necrosis factor α (TNF α), FAS ligand (FASL) and TRAIL, as well as to intracellular damage, upon the induction of pro-apoptotic BH3-only members of the Bcl-2 family. However, it is now clear that apoptosis is not the only cellular mechanism that mediates programmed cell death. Necrotic cell death, which has traditionally been viewed as a form of passive cell death, may also be regulated, and in this case has been called necroptosis or programmed necrosis.² Necroptosis may be induced by TNF α receptor 1 (TNFR1) agonists, but also by innate pattern-recognition receptors (PRRs) such as Toll-like receptor (TLR) 3 and TLR4.^{1,4} These two TLRs can recruit the adaptor TICAM-1 (also known as TRIF), leading to Type I interferon (IFN) signaling.³ In line with this notion, the TLR3 ligand polyI:C (a synthetic double-stranded RNA, dsRNA) can activate either apoptosis or necrosis, depending on the cell lines tested. Cell death induced by the TLR3-TICAM-1 axis may therefore be executed through two distinct subroutines.⁵ The mechanisms that dictate the cellular decision to undergo apoptosis or necroptosis in response to TLR3 signaling, as well as the mechanisms that mediate the execution of necroptosis, are the subject of intense investigation.

Toll-like receptors and other PRRs harbor the ability to specifically recognize microbial molecules, known as pathogen-associated molecular patterns (PAMPs).⁶ PAMPs trigger the maturation of myeloid dendritic cells (mDCs) through the activation of TLR and/or other pathways, eventually eliciting cellular immunity.⁷ In mDCs, nucleic acid-recognizing TLRs (i.e., TLR3, TLR7, TLR8 and TLR9) reside in endosomes and sense their ligands only when they are internalized.⁸ The uptake of DNA or RNA of microbial origin therefore allows cross-presentation to T cells and the exposure of natural killer (NK) cell-activating ligands. Besides this extrinsic maturation route, it is known that the formation of autophagosomes may deliver cytoplasmic nucleic acids of viral origin to the endosome via autophagy.⁹ In either route, TLR signaling links immunological events to pathological cell death.

Recently accumulated evidence suggests that TLRs serve as receptors not only for foreign PAMPs but also for cellular

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Table 1. Host response to nucleic acids and other DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids(PAMP)	
cytosolic long dsRNA	MDA5
cytosolic 5'-PPP-RNA	RIG-I
endosomal >140bp dsRNA	TLR3
nonmethylated CpG DNA	TLR9
cytosolic dsDNA	DNA sensors*
Self molecular patterns(DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4,**
S100 proteins	RAGE
Self nucleic acids (DAMP)	
Self DNA	DNA sensors*
Self mRNA	TLR3

*See Table 2; ** D40, CD91, Scavenger receptors etc.

constituents that are liberated from damaged or necrotic cells.¹⁰ Thus, innate pattern-recognition is not only a mechanism for discriminating pathogens from the host, but also a means for inspecting cellular homeostasis. Molecules that, upon release from damaged/necrotic cells, activate the immune system are called damage-associated molecular patterns (DAMPs).¹¹ The most popular TLR adaptor MYD88 is known to contain death domains, and some reports have suggested that TLR signaling may be involved in cell death secondary to PAMP/DAMP-stimulation. Necroptotic or damaged cells may thus represent a result of TLR death signaling, and generate a functional complex consisting of sources of DAMPs as well as of the phagocytic response.^{11,12}

DAMPs refer to intracellular molecules that acquire inflammation-inducing capacities when released from cells. DAMPs do not belong to the cytokine family but rather resemble PAMP in their functional properties, in particular with regard to mDC and macrophages. The functions of DAMPs may be associated with responses including regeneration and tumorigenesis. During the past 5 y, necroptotic cell death has been closely connected with innate immune responses involving pattern-sensing.^{12,13} DAMPs include a large number of cytosolic or nuclear molecules (Table 1), as well as, surprisingly, self nucleic acids.¹⁴ This implies that, like viral DNA and RNA, autologous nucleic acids can evoke inflammation. Here, we discuss the importance of the immune modulation induced by nucleic acids and necroptotic host cells.

Necroptosis: Programmed Necrosis Induced by TNF α

TNF α has been reported to induce two different types of cell death, apoptosis and necrosis, in a cell type-specific manner.^{15,16} Through TNFR1, TNF α is implicated in NF κ B activation and contributes to cell growth in many cancer cell lines. In parallel TNF α -induced hemorrhagic necrosis has been observed in

Table 2. RNA-DNA recognition molecules in vertebrates

Receptors	Adaptors	Ligands	Induction of Type I IFN
TLR family			
TLR3	TICAM-1	dsRNA, stem RNA	+
TLR7/8	MyD88	ssRNA	+
TLR22	TICAM-1	dsRNA	+
PKR	?	dsRNA	-
RLR family			
RIG-I	MAVS	5'-PPP RNA, dsRNA	+
MDA5	MAVS	dsRNA (long)	+
NLR family			
NALP3	ASC	dsRNA	+
NOD2	MAVS	ssRNA	+
DDX family			
DDX1	TICAM-1	dsRNA	+
DDX21	TICAM-1	dsRNA	+
DHX36	TICAM-1	dsRNA	+
DNA sensors			
TLR9	MyD88	CpG DNA	+
DAI	TBK1	dsDNA	+
Pol3/RIG-I	MAVS	dsDNA	+
IFI16	TBK1	dsDNA	+
DDX41	STING	dsDNA	+
DHX9	MyD88	dsDNA	+
DDX36	MyD88	dsDNA	+
ZAPS	?	dsDNA	+

several cancer cell lines, but the molecular mechanisms underlying these differential responses to TNF α remain poorly understood. Recently, several reports have suggested that the formation of a supracomplex containing the receptor-interacting protein kinase 1 (RIP1) and its homolog RIP3 (which has been named "necrosome") is responsible for the switch from apoptosis to necroptosis.^{17,18} RIP1 and RIP3 can assemble only in the absence of functional caspase-8, indicating that this enzyme acts as a key protease for blocking the formation of the necrosome.^{5,19} Many viral factors, as well as the genomic instability that frequently characterizes tumor cells, can compromise caspase-8 function, thereby facilitating the induction of necroptosis. Hence, TNF α can promote cell death by signaling through its receptors, including TNRF1 and downstream via RIP1/RIP3, although the output of TNF α signaling is ultimately determined by cell type.

Virus-mediated Necroptosis

It is notable that a necrotic phenotype has been observed in polyI:C-stimulated bone marrow-derived murine macrophages and other cell lines.¹³ TICAM-1 and RIP3 are involved in this process, suggesting the implication of the necrosome pathway in dsRNA-mediated cell death.^{12,13} It has been reported that viral

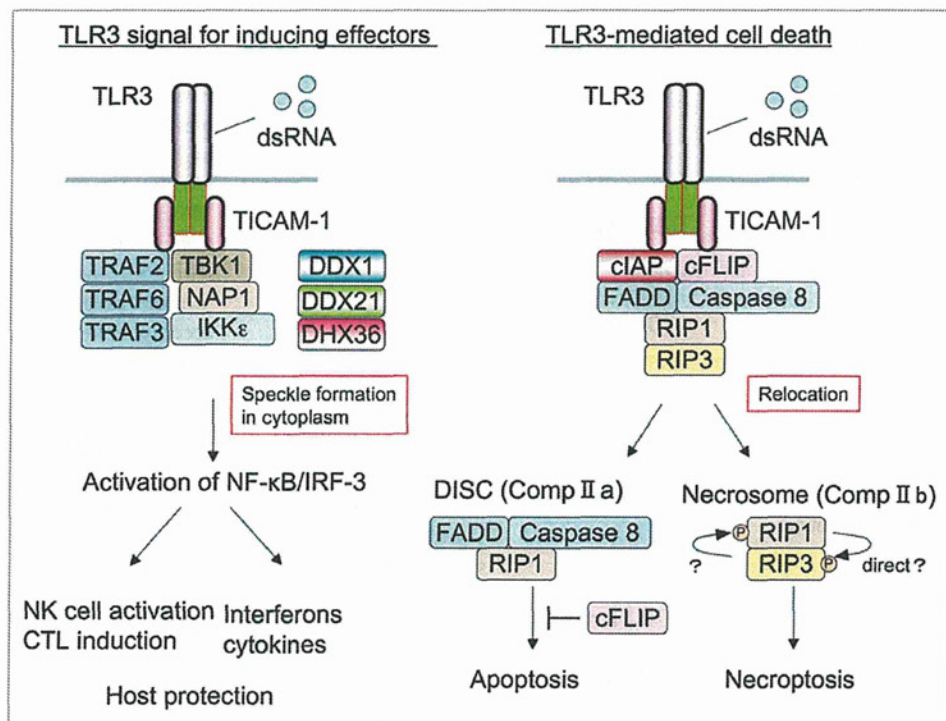


Figure 1. TLR3 signals inducing cell death or effector functions in myeloid cells. Cell survival (left panel) and cell death (right panel) signals are schematically depicted. TICAM-1 assembles in a supramolecular complex around oligomerized Toll-like receptor 3 (TLR3) in the endosome. The complex (named Speckle) then dissociates from TLR3, translocating to the cytoplasm. IRF-3 and NF κ B are activated by Speckle, leading to their nuclear translocation and induction of Type I interferon (IFN) and inflammatory cytokines, respectively. In dendritic cells (DCs), natural killer (NK) cell-activating ligands and factors for cross-presentation are induced downstream of IRF-3/7 (left panel). In contrast, cell death signaling culminates in apoptosis and/or necrosis depending on downstream signal transducers (right panel). TLR3-dependent apoptosis has been reported in several cancer cell lines,⁴ while TLR3-dependent necroptosis has been observed in mouse bone marrow-derived macrophages.¹³ These events rely on RIP1/RIP3 activation, similar to those elicited upon ligation of the tumor necrosis factor α receptor 1 (TNFR1). Whether or not the translocation of the TICAM-1 complex is required for the cell death signaling, as well as the mechanisms determining either cytokine secretion or cell death, remain unknown.

dsRNA frequently induces apoptosis in infected cells, a process that in general is known as cytopathic effect.²⁰ TICAM-1 and RIPs, mainly RIP1, may also be involved in virus-derived necrotic cell death.^{5,13} This is relatively rare compared with apoptosis since it occurs only when the viral genome encodes caspase-8 inhibitors.¹⁹ Furthermore, this process requires viral dsRNA to be delivered from the cytosol to the endosomes (where TLR3 is situated) of infected cells. This may happen if the dsRNA is engulfed by autophagosomes, which ensure its transfer to endosomes. The possible involvement of another PRR that sense viral RNA, RIG-I/MDA5, in cell death as induced by viral infection cannot be always ruled out. TNF α can be produced downstream of the TLR3- and RIG-I-mediated RNA-sensing pathways and may induce necrotic cell death,²⁰ but the factors determining the induction of necroptosis in virus-infected cells remain to be clarified.

DNA viruses can induce necroptosis via another mechanism, which involves the DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1/ZBP1).²¹ DAI is a DNA sensor²² and directly activates RIP3 in the absence of Type I IFN induction.²¹ This said, the sensing of DNA in the cytoplasm of virus-infected cells is complex, and it may be that DAI is not

the only molecule linked to such a necroptotic response. It is unknown whether RIP3-mediated necroptosis can be induced even if caspase-8 is blocked upon the recognition of viral DNA by DAI or via other mechanisms.²⁰ In fact, this type of virus-derived necrosis has been reported with DNA viruses that encode caspase inhibitors including vaccinia virus (VV), which encodes B13R/Spi2, poxvirus, encoding CrmA, the Kaposi's sarcoma-associated herpesvirus (KSHV), encoding K13 and the molluscum contagiosum virus (MCV), which encodes MC159.^{20,23} Generally speaking, the mode of cell death secondary to virus infection differ as a function of viral species. The physiological role of TLR3- and DAI-mediated necroptosis should therefore be analyzed in a virus-specific fashion.

Necroptosis in Inflammation

Apoptosis plays a major role in physiological contexts, while necrosis is very common under pathological conditions.¹ Necroptosis differs from accidental necrosis in its programmed nature, and differs from apoptosis in that necroptosis often stimulates inflammation. When virus-infected cells undergo apoptosis, they are removed by phagocytosis. Viral genomes, be they either

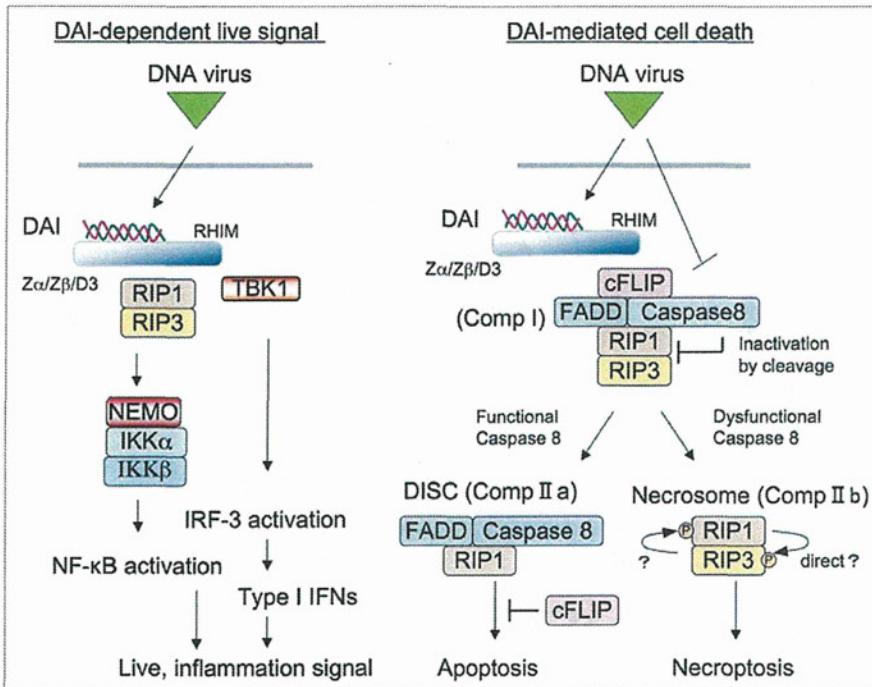


Figure 2. Necroptosis induced by the DAI pathway. Cell survival (left panel) and cell death (right panel) signals transmitted by the DNA-dependent activator of IFN-regulatory factors (DAI) are schematically depicted. Pro-survival signaling involves the activation of IRF-3 and NFκB to support antiviral responses (left panel). Type I IFNs and inflammatory cytokines are the main effectors induced by IRF-3/NFκB activation. In contrast, DAI activates RIP3 to induce necroptosis during viral infection, provided that caspases are inhibited. When viruses express caspase inhibitors, the RIP1/RIP3 necrosome plays a dominant role in the activation of cell death via necroptosis (right panel). If caspase-8 is active, RIP3 should get inactivated and apoptosis should be the dominant phenotype, though this scheme has not yet been experimentally confirmed. The mechanisms determining the choice between these two signaling pathways are unknown.

DNA- or RNA-based, are degraded in infected cells, thus being able neither to stimulate phagocytes including macrophages and DCs, nor to favor the liberation of DAMPs. In contrast, non-apoptotic cell death is accompanied by the release of DAMPs and viral products, resulting in the activation of macrophages,¹³ as it occurs during chronic infection, in which viruses produce caspase inhibitors or render infected cells resistant to apoptosis.²⁴ A typical model of necroptosis evokes two effectors, namely, viral nucleic acids and DAMPs, to modulate immune and bystander cells of the host. In the context of necroptosis, these effectors allow for the amplification of inflammatory responses by myeloid phagocytes (mDCs and macrophages). These cells accumulate in inflammation as induced by persistent viral infection, and mediate the secondary release of cytokines and other biologically active molecules. In addition, viral factors can result in incipient inflammation, as observed in chronic infections by the hepatitis B or C virus.²⁴ This, in conjunction with viral nucleic acids and DAMPs, may modify the features of the infectious milieu. Further studies are needed to clarify the importance of viral nucleic acids and DAMPs in the context of virus-dependent chronic inflammation, as it may facilitate tumor progression.

Necroptosis and Oncogenesis

Accumulating evidence indicates that pro-inflammatory signals, including those following the activation of NFκB, are crucial for oncogenesis. Moreover, DAMPs have been associated with tumorigenesis as well as with antitumor immune responses.^{25,26} Tumor progression is not always accompanied by viral infections, and it remains unclear whether DAMPs released from non-infected tumor cells are sufficient to support tumor growth. It has been reported that self mRNA acts as a TLR3 ligand¹⁴ and that self DNA can stimulate host cell sensors.^{22,27} Due to the uncomplete identification and functional characterization of DNA sensors and their signaling pathways, however, it is unknown whether host nucleic acids are potent inducers of inflammation as compared with viral RNA or unmethylated CpG DNA of bacterial origin. Moreover, the role of RNA sensors in the tumor microenvironment has not yet been clarified (Table 2).

DAMPs have recently been characterized at the molecular level¹¹ and representative DAMPs (Table 1) include HMGB1,²⁸ uric acid crystal,¹⁰ S100 proteins,²⁹ naked actin^{30,31} and heat-shock proteins (HSPs).³² The functional features of DAMPs and the mechanisms whereby they provoke inflammation have been delineated,^{11,28,29} and these studies have introduced the concept of “inflammasome” in the field of innate immunity.³³ Caspase-1 is activated upon the administration of NOD-like receptor (NLR) ligands, which include some DAMPs as well as inorganic PAMPs. Active caspase-1, together with the upregulation of the immature variants of IL-1 family proteins that ensues TLR stimulation, accelerates the robust release of IL-1β, IL-18 and IL-33.³⁴ There are many kinds of NLRs as well as TLRs, and the common pathways (including those centered around the adaptor ASC) can be activated by a variety of cytoplasmic DAMPs and PAMPs.^{33,34} The cytoplasmic immature forms of the abovementioned cytokines are activated by limited caspase-1-mediated proteolysis, and then are secreted into the extracellular microenvironment.³⁴ Hence, IL-1 family proteins require two DAMPs/PAMP signals for their upregulation and activation.³⁵ Of note, the tumorigenic properties of asbestos and silica are in part attributable to the activation of the inflammasome, leading to the secretion of IL-1 family proteins. However, not all DAMPs operate as inflammasome activators, even in the broad sense of this term.

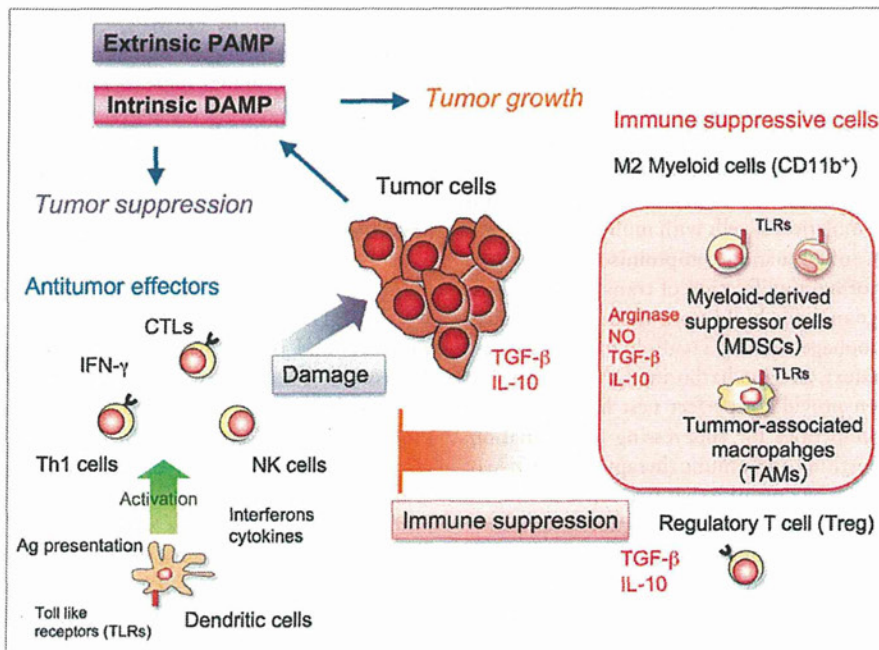


Figure 3. Inflammation provides the microenvironment for infection-related cancer. Immune cells infiltrating the tumor mass may modulate the local microenvironment upon the recognition of pathogen- or damage-associated molecular patterns (PAMP/DAMPs). Cancer cells undergoing necrosis liberate DAMPs and debris containing nucleic acids, which recruit immune cells stimulating an inflammatory response. In some cases, tumors benefit from the inflammatory response, while in other cases they regress following inflammation. The mechanisms determining this switch remain to be clarified.

Immune Response Elicited by the Phagocytosis of Dead Cells

Phagocytosis of dead cells involves not only cell clearance but also the initiation of an immune response. Dead cell antigens are rapidly presented on MHC Class II molecules after internalization by DCs, driving the recruitment and activation of various CD4⁺ T cell subsets, including Th1, Th2, Th17 and regulatory T cells (Tregs) (Fig. 1). In the presence of a second co-stimulatory signal provided by TLRs, working as an adjuvant, DCs cross-present antigens on MHC Class I molecules to induce the proliferation of CD8⁺ cytotoxic T lymphocytes (CTLs).³⁶ The presentation of exogenous antigens by DCs is therefore dependent on the presence of PAMPs/DAMPs.³⁶ Accordingly, necrotic debris appears to result in CTL cross-priming more efficiently than apoptotic bodies. Cross-presentation is enhanced by molecules such as Type I IFN and CD40, and by immune cells including CD4⁺ T, NK and NKT cells. Hence, the use of adjuvants to affect many cell types of the immune system other than antigen-presenting cells, and a precise evaluation of the total cross-priming activity appear to be indispensable for the development of efficient adjuvant therapies.

The TLR3/TICAM-1 axis is best known as an inducer of cross-presentation *in vivo*.³⁷ The cross-presentation activity of the TLR3 ligands polyI:C and viral dsRNA was first described by Schulz et al. in 2005.³⁸ While the potency of polyI:C as an adjuvant has been reported by Steinman and colleagues,^{37,39} the precise identity of the DAMPs participating in cross-presentation

and possessing latent cross-priming (CTL-inducing) capacities has not yet been determined.

It is known that phagocytosis induces functional changes in mDCs and macrophages (Fig. 2): phagocytes are skewed toward a regulatory phenotype accompanied by the production of IL-10 and TGFβ during the phagocytosis of apoptotic cell debris, even in the presence of PAMP.^{40,41} This suggests that material that cannot be taken up exerts different effects on mDCs than internalizable material during their phagocytic interactions. Phagocytes undergo cytoskeletal rearrangement when they take up cell debris, involving cell adhesion molecules that accelerate the interaction between the phagocyte membrane and cell debris. The opsonization of dead cells further enhances phagocytosis as well as the induction of an immune outcome.⁴² Complement-mediated opsonization of dead cells strongly alters the functional properties of mDCs and macrophages.⁴³ Yet, it has been impossible to discriminate apoptotic and necroptotic cells based on this.⁴⁴ Thus, the mechanisms whereby necroptotic cells initiate an immune response upon phagocytosis by mDCs and macrophages, compared with apoptotic cells, remain largely uncharacterized. Elucidating the role of necroptotic cells and DAMPs as adjuvants for NK-cell activation and antigen presentation is highly relevant for antitumor therapy. Since the phagocytosis of dead cells by mDCs usually leads to the generation of tolerogenic mDCs, additional adjuvants appear to be required for mDCs to present tumor antigens in an immunogenic fashion, leading to the induction of an effective immune response against cancer.

Termination of Inflammation

Inflammation often drives tissue repair and regeneration, and the microenvironment formed during inflammation serves as a basis for assembling cells that initiate tissue development and reorganization (Fig. 3). The pro-inflammatory microenvironment facilitates cell growth as well as genome instability, thus being prone to the accumulation of cells with multiple mutations. Furthermore, incipient inflammation compromises the immune system so that the abnormal proliferation of transformed cells is tolerated. Thus, malignant cells build up a tissue that involves tumor-associated macrophages serving a scaffold for invasion and metastasis.⁴⁵ In this context, a region harboring DAMP-mediated persistent inflammation provides a perfect nest for tumor progression (Fig. 3). Therapeutics for suppressing inflammation, such as aspirin, may constitute an immune therapy irrespective of the presence of infection.⁴⁶ We surmise that two types of inflammation exist, namely tumor-supporting and tumor-suppressing, implying that inflammation is a complex phenomenon consisting of multiple distinct aspects. We have shown that some adjuvants can induce tumor-suppressing inflammation, thereby limiting

tumor proliferation by DAMPs.⁴⁷ The adjuvant-induced switch of cell death/inflammation signals to an antitumor outcome is an intriguing approach for cancer therapy, particularly in view of the fact that the mechanisms of adjuvant signaling are being increasingly characterized at the molecular level.^{48,49} The clarification of the role of adjuvant signaling in compromising tumor progression will lead to the discovery of non-toxic synthetic tumor-regressing molecules with potential as novel anticancer therapeutics.⁵⁰

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TAMable tumor-associated macrophages in response to innate RNA sensing

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Key words: TLR3, TICAM-1, tumor-infiltrating macrophages, polyI:C, immunotherapy

Antitumor effect of PolyI:C (a viral dsRNA analog) has been attributed to dendritic cell (DC)-maturation activity, that drives antitumor NK cells, DC cross-presentation, cytotoxic T lymphocytes and many IFN-inducible genes. According to a recent paper, tumor-infiltrating M2 macrophages are found to become an additional antitumor effector through polyI:C response.

Interferon (IFN), now categorized as type I, was discovered by Isaacs and Lindeman in 1957. Soon after their discovery, it was expected to be a fascinating medicine opposing to virus infection and cancer development. Type I IFN inducing activity was assigned to the signature of double-stranded RNA generated from viruses, and its synthetic analog, polyI:C, was confirmed to serve as an effective inducer of type I IFN. Talmadge et al. showed that polyI:C mixed with polyL Lysine and methylcellulose (polyI:CLC) effected dramatic regression of syngenic implant tumors in mice. They suggested this reagent might be applied to antitumor therapy. In line with these reports, there have been many reports indicating that spontaneous tumor regression sometimes occurs in cancer patients when they are exposed to viruses or viral vectors.

PolyI:C induces type I IFN and inflammatory cytokines. In addition, it may contribute to raising cellular immunity. According to recent progress in pattern recognition of innate immunity, polyI:C is a ligand for multiple receptors, including PKR, RIG-I, MDA5 and TLR3.² Virus replication usually amplifies dsRNA production inside the cytoplasm of affected cells and stimulates the cytoplasmic RNA sensors. In contrast, TLR3 is activated when dsRNA generated in infected cells is released and internalized into the endosome of bystander

phagocytes,² such as dendritic cells (DC) and macrophages. dsRNA is delivered through a unique pathway involving Raftlin,³ then the endosomal TLR3 passes the signal to the adaptor TICAM-1.² The multiple functionality of polyI:C may reflect its divergent receptor usage, and knockout mouse (KO) studies have therefore been indispensable for determination of the role of each receptor in antitumor immunity.

In mouse models, growth retardation of syngenic implanted tumor has been reportedly observed by administration of polyI:C, which is now attributable to liberated type I IFN and maturation of DC, that drives NK and killer T cells.^{4,5} The mechanisms whereby these effector cells are introduced by dsRNA are being elucidated on a molecular level: the TLR3/TICAM-1 pathway for dsRNA recognition in DC is involved in effector driving. In a recent paper, Shime et al. additionally identified the third antitumor effector induced by ip polyI:C administration.⁶ PolyI:C acted on tumor-infiltrating macrophages and induced tumor growth retardation in some tumor species. Administration of polyI:C rapidly (< 12 h) led to tumor hemorrhagic necrosis followed by tumor regression. The results appear to resemble an earlier report by Old's group on the TNF α -mediated fibrosarcoma regression.⁷ In fact, TNF α participated in hemorrhagic necrosis in this

case also. Shime et al. applied KO mice models for analyzing the signaling pathway by which the polyI:C-derived tumor regression occurs. Ultimately, their conclusion was that tumor-infiltrating macrophages (Mf) characterized by CD11b⁺/F4/80⁺/Gr-1^{low} markers with sustaining tumor-supporting phenotype, M2, serves as a target for polyI:C and changes their properties to antitumor, M1-like, behaving like a tumoricidal effector. In these Mf, TLR3/TICAM-1 pathway, but not the IPS-1 pathway, is also mandatory for TNF α production and tumor regression. Indeed, the marker profile of the Mf was similar to those reported as M2 Mf or tumor-associated Mf (TAM). It is notable that they have high expression levels of TLR3. Hence, the polyI:C tumor growth retardation is mechanically multifarious and involves TNF α hemorrhagic necrosis.

TLR3 is highly expressed in CD8⁺ splenic DC and CD103⁺ non-lymphoid DC in mice,⁸ and they are strong inducers for cross-priming of CD8 T cells,^{5,8} namely cytotoxic T lymphocytes (CTL). TLR3-positive bone marrow-derived DC also reportedly induce type I IFN and potent antitumor NK cell activity.⁴ Thus, polyI:C functions through TLR3⁺ myeloid cells to facilitates antitumor cellular immunity encompassing at least three distinct routes, NK cell activation, CTL proliferation and conversion of TAM to an tumoricidal effector (Fig. 1). Hence,

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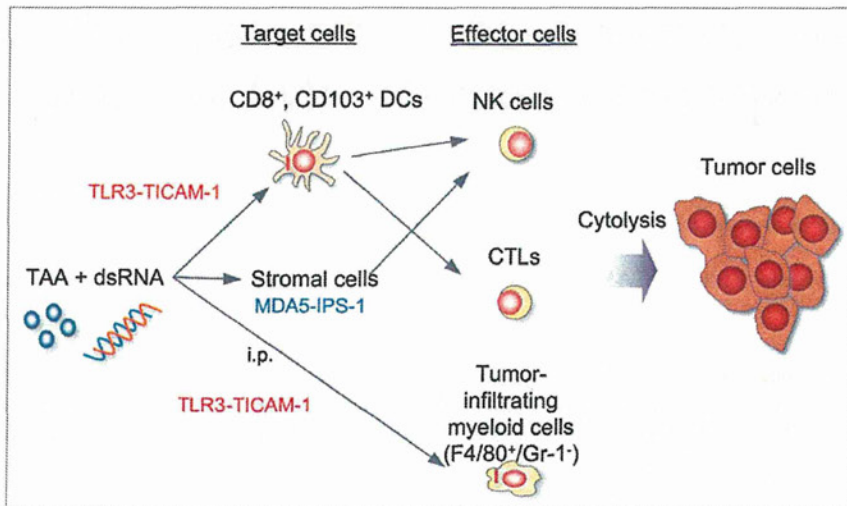


Figure 1. PolyI:C induces three antitumor effectors via different routes. Antitumor activity of polyI:C against tumor cells are assessed in mouse tumor-implant models. A unique point in this review is the third pathway where tumor-infiltrating myeloid cells are involved, effectively damages Lewis Lung carcinoma cells. This tumoricidal activity is mediated by the TICAM-1 pathway in the myeloid cells, and attributed to TNF α . Although polyI:C is i.p. administered, it acts on tumor-infiltrating Mf and converts them to antitumor effectors.

the Janeway/Medzhitov concept⁹ may be adaptable to tumor immunology that pattern recognition receptor (PRR) stimulation by a specific ligand triggers innate immune response and facilitates establishment of the cellular immune system.

A tantalizing reagent for successful peptide vaccine therapy against cancer using tumor-associated antigens (TAA) with CD4/CD8 epitopes is adjuvant. Nevertheless, polyI:C therapeutic use has been very restricted in patients. This is because polyI:C has severe side effects, enterocolitis, arthralgia, fever, erythema and sometimes life-threatening hypotonic shock, which have prevented the clinical use of this dsRNA analog. However, a recent study reported that polyI:CLC is applicable to humans, although robust erythema and cytokine upregulation in serum are usually accompanied as side effects with expected therapeutic potential.¹⁰ Dr. Steinman, having won the Nobel prize, proposed a polyI:C/TAA therapy for cancer patients if the TAA is identified in each case of the patients. Shime's data confirmed this

issue and further clarified the importance of the TICAM-1 pathway in triggering induction of antitumor Mf in addition to NK cells and CTL.⁶ These sequential studies, together with the direct apoptotic effect of polyI:C on tumor cells, reinforce the need to establish a safer RNA derivative for human immunotherapy in the future.

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JB Review

Ubiquitin-mediated modulation of the cytoplasmic viral RNA sensor RIG-I

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RIG-I-like receptors, including RIG-I, MDA5 and LGP2, recognize cytoplasmic viral RNA. The RIG-I protein consists of N-terminal CARDs, central RNA helicase and C-terminal domains. RIG-I activation is regulated by ubiquitination. Three ubiquitin ligases target the RIG-I protein. TRIM25 and Riplet ubiquitin ligases are positive regulators of RIG-I and deliver the K63-linked polyubiquitin moiety to RIG-I CARDs and the C-terminal domain. RNF125, another ubiquitin ligase, is a negative regulator of RIG-I and mediates K48-linked polyubiquitination of RIG-I, leading to the degradation of the RIG-I protein by proteasomes. The K63-linked polyubiquitin chains of RIG-I are removed by a deubiquitin enzyme, CYLD. Thus, CYLD is a negative regulator of RIG-I. Furthermore, TRIM25 itself is regulated by ubiquitination. HOIP and HOIL proteins are ubiquitin ligases and are also known as linear ubiquitin assembly complexes (LUBACs). The TRIM25 protein is ubiquitinated by LUBAC and then degraded by proteasomes. The splice variant of RIG-I encodes a protein that lacks the first CARD of RIG-I, and the variant RIG-I protein is not ubiquitinated by TRIM25. Therefore, ubiquitin is the key regulator of the cytoplasmic viral RNA sensor RIG-I.

Keywords: RIG-I/type I interferon/ubiquitin/virus.

Abbreviations: CARD, caspase activation and recruitment domain; CTD, C-terminal domain; dsRNA, double-stranded RNA; RLR, RIG-I-like receptor; pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; MEF, mouse embryonic fibroblast cell; BM, bone-marrow; Mφ, macrophage; IFN, interferon; ISG, interferon-stimulated gene; TRIM, tripartite motif; RNF, RING finger.

Recognition of viral RNA

Type I interferons (IFNs) are inflammatory cytokines that possess strong anti-viral activity. During viral infection, type I IFNs are produced from dendritic cells (DC), macrophages (Mφ) and fibroblast cells (Fig. 1A). Viral RNA is mainly recognized by Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). TLRs are

type I transmembrane proteins. TLR3, 7 and 8, which are members of the TLR family, are localized to endosomes, and are responsible for the recognition of viral RNA (1). RLRs are DExD/H box RNA helicases and recognize viral RNA in the cytoplasmic region (Fig. 1B). There are three members of the RLR family: RIG-I, MDA5 and LGP2. RIG-I has the ability to recognize various types of viruses, and MDA5 mainly recognizes picornaviruses (2). LGP2 promotes RIG-I and MDA5-mediated signalling (3).

A cytoplasmic sensor for the detection of viral RNA

RIG-I, a cytoplasmic sensor for viral RNA, is induced by viral infection, polyIC and type I IFN stimulation (4). This protein is composed of two N-terminal caspase recruitment domains (CARDs), a central DExD/H box helicase/ATPase domain and a C-terminal regulatory domain (CTD) (Fig. 2). N-terminal CARDs are responsible for the binding to the adaptor molecule IPS-1/MAVS/VISA/Cardif, which is located on the outer membrane of the mitochondria (5–8). In the absence of viral RNA, RIG-I CTD represses the interaction between RIG-I CARDs and IPS-1 CARD (9). RIG-I CTD recognizes the 5' triphosphate of short double-stranded RNA, leading to multimerization of RIG-I and IPS-1 (10–13). IPS-1 triggers signaling to induce type I IFN and other inflammatory cytokines through STING (also called MITA) protein, which is localized to the endoplasmic reticulum or the mitochondria (14–17). STING then activates transcription factors, such as IRF-3, IRF-7 and NF-κB (15, 18).

Knockout of RIG-I abrogates the production of type I IFNs and inflammatory cytokines from mouse embryonic fibroblasts (MEFs), conventional DC and Mφs in response to viral infections, including infections caused by vesicular stomatitis virus (VSV), Sendai virus (SeV), influenza A virus, Newcastle disease virus, hepatitis C virus and Japanese encephalitis virus (2, 19). However, RIG-I is not necessary for the production of type I IFNs by plasmacytoid dendritic cells (pDCs), which are strong inducers of type I IFNs *in vivo* (19). In pDCs, TLR7 is responsible for the detection of viral RNA (20). In addition, knockout of IPS-1 and STING inhibits the production of type I IFNs from MEFs, Mφs and cDCs, but not from pDCs (15–18). Once type I IFNs are produced from these cells, IFN production is secondarily amplified via the IFNAR (21). The deficiency of the RIG-I-dependent pathway causes a reduction in early type I IFN production *in vivo* but shows only a marginal effect on late type I IFN production (15–18). Knockout of RIG-I increases the

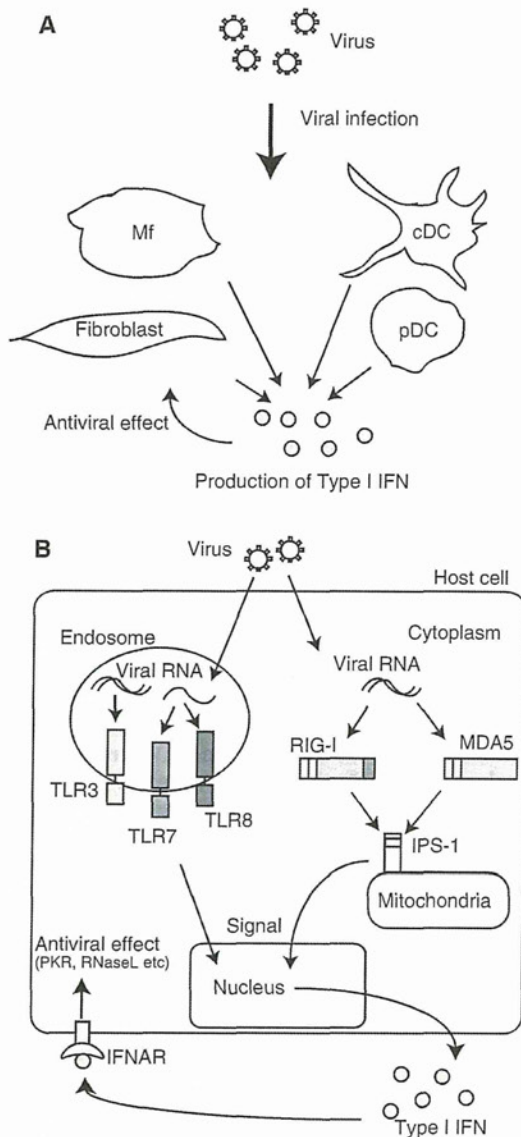


Fig. 1 Production of type I IFN in response to viral infection. (A) Type I IFN is a cytokine that possesses strong anti-viral activity. Type I IFN is produced from fibroblast cells, cDC, pDC and Mf in response to viral infection. (B) TLR3, 7 and 8 are localized to endosomes and are responsible for the 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.

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)

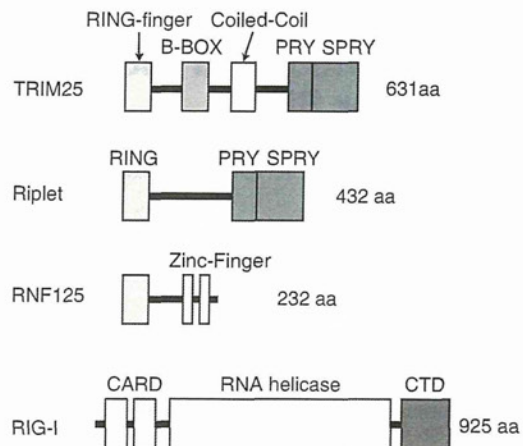


Fig. 2 Domain structures of TRIM25, Riplet, RNF125 and RIG-I. TRIM25 consists of RING finger, B-box, coiled-coil, PRY and SPRY domains. Riplet is similar to TRIM25 and consists of RING-finger, PRY and SPRY domains. RNF125 consists of RING-finger and two zinc-finger domains. Three proteins mediate the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

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-1 β into mature, bioactive IL-1 β (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).

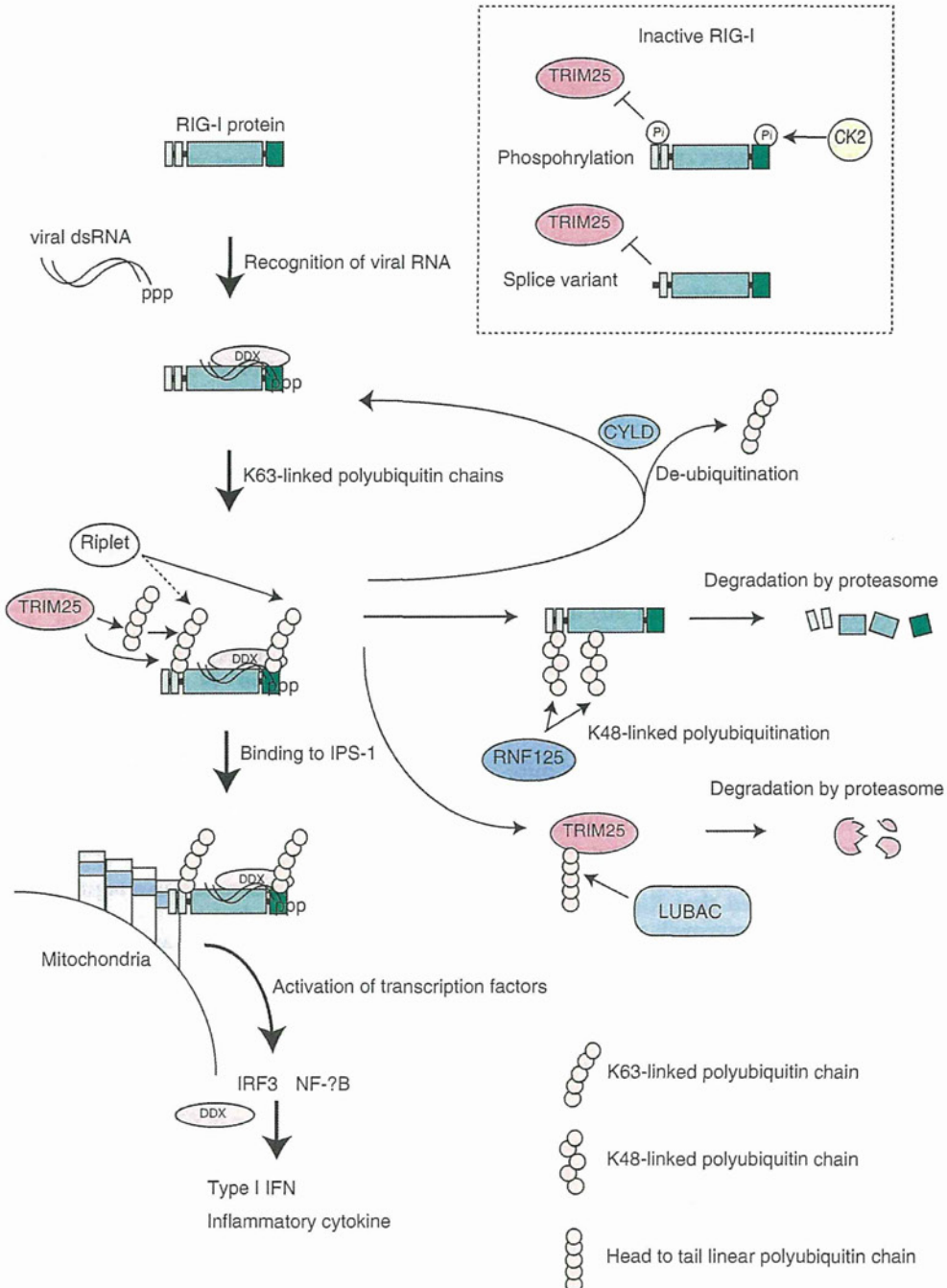


Fig. 3 Regulation of RIG-I by the ubiquitin chain. RIG-I binds to viral RNA together with other cofactors, such as DDX3. After the recognition of viral RNA, RIG-I changes its conformation and harbours K63-linked polyubiquitination by TRIM25 and Riplet. Polyubiquitination causes the activation of IPS-1, 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 polyubiquitination 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- κ B activation by RIG-I, which is required for IL-1 β mRNA expression; thus, knockout of TRIM25 reduces the production of mature IL-1 β (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- κ B 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 IKK ϵ , 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 sequesters 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 signalings, 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.

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Conflict of Interest

None declared.

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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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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.

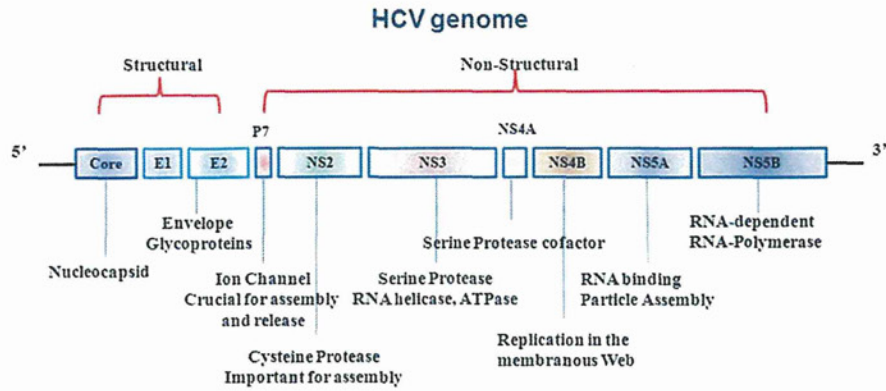


Fig. 1. Genomic structure of HCV. Genomic organization of wild-type HCV. The HCV-RNA genome consists of a major open reading frame, encoding a single polyprotein, and an alternative reading frame encoding F-proteins with unknown functions. The cleavage of the polyprotein by viral and host cell proteases 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- α (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.