signaling and liberates damage-associated molecular patterns (DAMP) of autologous TLR3 ligand (**Table 2**). Levels of RNA-derived TNF-α and its receptor, TNFR1, have been implicated in this process [74]. The RIP1/RIP3 complex, termed the necrosome, is responsible for switching between apoptosis and necroptosis [75,76]. TICAM-1 and RIP1 may be involved in the virus-derived as well as tumor cytolysis [77], although the possible involvement of RIG-I/MDA5 in cell death cannot be ruled out in some cases of viral infection [78]. DAMP and stemmed RNA can be liberated from tumor-infiltrating Mf as well as necroptotic tumor cells [77]. TNF-α and IL-6 are the pro-inflammatory cytokines released from Mf. A reported feature of exogenous dsRNA in the context of the tumor environment is to damage tumor cells by activation of Mf or the TLR3 pathway in these cells [10]. However, in tumor microenvironment containing tumor-infiltrating Mf, the role of the endogenous stemmed RNA in tumor progression and immune cell activation is the next issue to be elucidated (**Figure 2**).

7. Cellular immunity induced in tumor microenvironment

Once DC or Mf responds to an unusual innate dsRNA signature, cellular immunity is provoked against tumor cells with irregular modification of RNA-sensing pathways by these immune enhancers (**Figure 2**). NK cells and CTL are known to be associated with maturation of myeloid DCs after stimulation with dsRNA [79-81]. DCs express NK-activating ligands after recognizing dsRNA [82], and cell damage has been reported to play a role in the regulation of NK-activating ligands [83]. In this manner, dsRNAs are involved in tumor damage secondary to activation of cellular effectors. Subsequently, TLR3-stimulated DCs modulate cross-priming of CD8 CTLs through incorporation of dsRNA and Ag-mounted cell debris [84]. FasL and TRAIL are major effectors for the ligands of death receptors (DRs) [85].

Soluble mediators also function as effectors in response to dsRNA. The tumor microenvironment contains many cell types and tissues, on which dsRNA and DAMP act to effect the immune response (**Figure 2**). Systemic administration of polyI:C induces type I IFN and enhances local T-cell immune responses in the lung and liver [48,86]. It has been postulated that polyI:C-induced type I IFN mediates the production of IL-7 [88], which promotes T-cell-derived IFN-γ to enhance macrophage recruitment and CXCR3 ligand expression [86]. NK cells are involved in early onset of IFN-γ in response to polyI:C [32] after which IFN-γ is then robustly released through IL-7 production. IL-7 is produced in the lung and liver in a type-I IFN- and IFN-γ-dependent fashion [86,87]. In addition, polyI:C-induced IL-7 promotes expression of MCP-1, contributing to recruitment of macrophages and production of CXCR3 ligands by these cells [73,88,89]. This role of polyI:C in the tumor environment defines a new mechanism by which tumor-infiltrating T/NK cells boost local T-cell immunity and by which IL-7 bridges TLR3 signal to adaptive immunity.

Our laboratory has reported that a dsRNA analog strongly activates NK cells *in vivo* [32]. Two main routes for NK cell activation have been reported. Firstly, DCs secrete several cytokines, such as IL-12, IL-18, IL-15, and IFN-α/β in response to dsRNA, and these mediators act on NK cells [33,90]. Secondly, DCs express NK-activating ligands on their cell surface which activate NK cells through cell cell contact [57]. In mouse studies, transacting IL-15 and cell-surface NK-activating ligands are crucial in polyI:C-mediated NK cell activation [33,57]. The primary NK-activating ligand induced by polyI:C is IRF-3-inducing NK-activating molecule (INAM), which contributes to NK-sensitive tumor regression [57]. In a human system with BMDC and HCV-infected debris (a source of dsRNA), NK cells are activated by BMDC via the TLR3-TICAM-1 pathway in BMDC [82]. Based on these observations, INAM may therefore participate in dsRNA-derived NK activation. It is notable that the minimal dose of dsRNA for NK activation is higher than that required for induction of type I IFN in *in vivo* systemic administration studies.

RNA-derived molecular patterns of DAMP may cause TLR3-mediated inflammation resulting from physicochemical stimuli (**Figure 2**). However, the functional properties of stemmed RNA generated in tumor-related inflammation have not been well demonstrated [38]. Once antigens are presented on MHC class II in DCs upon internalization of tumor cell debris, CD4 T cells [91,92], including Th1, Th2, Th9, Th17, and Tregs, are driven in a context-dependent manner. Stemmed RNA and DAMP (**Table 2**) may act as modifiers of this event for CD4 T cells. The induction profiling of CD4 T-cell subsets critically affects the effector-inducing capacity of myeloid DC [91], although it remains unclear whether systemic type I IFN (and the MAVS pathway) is absolutely required or not for adaptive immunity. In addition, these stimulators may serve as the second signal of TLRs triggering DCs to induce cross-presentation, which leads to mounting Ag on MHC class I and subsequently induce the proliferation of CD8 T cells (CTL) [93]. Cross-presentation is enhanced by molecules such as type I IFN and CD40, and by immune cells, including CD4 T cells, NK cells, and NKT cells [93,94]. The mechanistic role of nucleic acids sensors in the presentation of exogenous Ag by DCs remains to be determined [61]. TLR3/TICAM-1 is the main pathway for inducing cross-presentation in response to dsRNA in DCs [34]. PolyI:C or virus dsRNA is an example of a TLR3 ligand, and the cross-presentation-inducing activity of these TLR3 agonists is noticeable if sufficient amounts of polyI:C are used [8]. While the effective adjuvancy of polyI:C has been reported by Steinman *et al.* [61,91,93], no report has definitively determined the dose of RNA sufficient to promote cross-presentation and latent cross-priming (CTL-inducing) ability in humans. Further therapeutic dose analysis will provide a basis for effective strategies of dsRNA

therapy in patients who do not respond to conventional cancer therapy.

8. Expert opinion

Here, we discussed the advantages of TLR3 agonists as a therapeutic potential against cancer. TLRs generally activate transcription factors, NF-κB, which closely associates with protumor activity, thereby application of TLR agonists to adjuvant immunotherapy for cancer treatment having been controversial. TLR3 is particular in the TLR family receptors because it is not involved in MyD88 activation but only in TICAM-1 for IRF-3/7 activation, which results in production of type I and III IFNs. TLR3 is localized to the endosomal membrane in mouse $CD8\alpha^+$ DC and human $CD141^+$ DC, suggesting that in viral infection, DCs phagocytoze noninfectious dsRNAs liberated from infected dead cells together with viral antigens. TLR3 in the DCs senses the internalized dsRNA to signal the IFN-inducing pathway. Epithelial cells and fibroblasts express TLR3 on the cell surface and directly sample dsRNA outside the cells, which may reflect the role of TLR3 in testing environment around the cells. Similar events might happen in tumor cells and DC surrounding microenvironment. Expression of TLR3 is up-regulated during malignant transformation, by eIF2 and RB, suggesting that many tumor cells can be modulated by their own TLR3 signal. The RIP1/3 pathway downstream of TICAM-1 can induce NF-κB activation, apoptosis or necroptosis that facilitates liberation of tumor antigen and its uptake by DC. Necroptosis secondary to RIP1/3 signal may be a representative outcome induced by tumor cell TLR3, although the protumor activity that induces tumor progression via the TLR3/TICAM-1 pathway is predicted to be negligible compared to the MyD88 pathway. Besides TLR3, RIG-I and MDA5 act as cytoplasmic sensors to induce systemic cytokine/IFN production leading to high serum cytokine levels. The most prominent side-effect induced by dsRNA or polyI:C (or LC) is a life-threatening cytokine shock. Indeed, the serum cytokine/IFN levels in WT mice treated with polyI:C are highly increased, but the levels are kept low in MAVS-/- mice, suggesting that polyI:C-mediated cytokinemia is largely attributable to the MAVS pathway. Although serum cytokines are high in TICAM-1-/- mice, NK cell activation and CTL proliferation are severely impaired in the absence of TICAM-1. Ultimately, we would predict that exclusive stimulation of TLR3 (i.e., TICAM-1) does not allow the serum cytokine/IFN levels in mice, whereas cellular immune effectors NK and CTL are sufficiently driven by TLR3-directed immunotherapy even in MAVS-/- mice. The strategies for specific targeting of TLR3 in dendritic cells without affecting MDA5/RIG-I should be developed for more efficient antitumor immunotherapy. If TLR3-targeted dsRNA therapy is established, tumor regresses without evoking either tumor progression or cytokinemia, two major side-effects by dsRNA-mediated inflammation then being cleared. If these TLR3 outputs are reproducible in human patients with cancer, dsRNA derivatives specifically directed against TLR3 will be an excellent therapeutic candidate for tumor immunotherapy as an adjuvant.

Clinical studies of polyI:CLC therapy for cancer was started on 1985. Since then, many clinical trials have been performed with polyI:C or LC. Most of them suggested that low doses of polyI:C did not always bring the patients good prognosis. This suggests that low-dose administration of dsRNA to patients, which appears sufficient for induction of type I IFN, is insufficient for induction for DC-driven NK activation and CTL proliferation. If administration of high doses of harmless dsRNA is feasible for adjuvant therapy, then patients with cancer benefit from therapeutic use of dsRNA. Development of less-toxic compounds specific for TLR3 would help patients with inoperable or drug-resistant tumors.

Declaration of interest

This manuscript was sponsored by grants from the Japanese Government, particularly by MEXT Grant-in-Project 'the Carcinogenic Spiral' and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID). The authors declare no other conflicts of interest and have received no payment in preparation of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest ( ) or of considerable interest ( ) to readers.

1. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8

•• **The first paper describing TLR3 as a dsRNA receptor.**

2. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085-8

• **The first paper describing the relationship between TLR4 mutation and endotoxin shock.**

3. Oshiumi H, Matsumoto M, Funami K, et al. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 2003;4:161-7

•• **This paper describes that direct interaction between TLR3 and TICAM-1 (TRIF) is essential for TLR3-mediated IFN signaling.**

4. Matsumoto M, Kikkawa S, Kohase M, et al. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Commun 2002;293:1364-9

• **The first introduction of human TLR3-function-blocking mAb.**

5. Yoneyama M, Kikuchi M, Natsukawa T, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730-7

• **This is the first paper showing that RIG-I is an cytoplasmic dsRNA sensor.**

6. Yoneyama M, Kikuchi M, Matsumoto K, et al. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 2005;175:2851-8

7. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441:101-5

8. Gitlin L, Barchet W, Gilfillan S, et al. Essential role of mda-5 in type I IFN responses to polyriboinosinic: polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci USA 2006;103:8459-64

9. Ermolaeva MA, Michallet MC, Papadopoulou N, et al. Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. Nat Immunol 2008;9:1037-46

10. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 2010;11:700-14

11. Shime H, Matsumoto M, Oshiumi H, et al. Toll-like receptor 3 signaling converts tumor-supporting myeloid cells to tumoricidal effectors. Proc Natl Acad Sci USA 2012;109:2066-71

12. Oshiumi H, Tsujita T, Shida K, et al. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, Fugu rubripes, genome. Immunogenetics 2003;54:791-800

13. Matsuo A, Oshiumi H, Tsujita T, et al. Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. J Immunol 2008;181:3474-85

14. Seya T, Matsumoto M, Ebihara T, Oshiumi H. Functional evolution of the TICAM-1 pathway for extrinsic RNA sensing. Immunol Rev 2009;227:44-53

15. Matsumoto M, Funami K, Tanabe M, et al. Subcellular localization of toll-like receptor 3 in human dendritic cells. J Immunol 2003;171:3154-62

• **This paper highlights the endosomal localization of DC TLR3 using the specific mAb.**

16. Johnsen IB, Nguyen TT, Ringdal M, et al. Toll-like receptor 3 associates with c-Src tyrosine kinase on endosomes to initiate antiviral signaling. EMBO J 2006;25:3335-46

17. Schulz O, Diebold SS, Chen M, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005;433:887-92

•• **This paper highlights the importance of TLR3 in DC cross-presentation.**

18. Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 2008;60:805-12

19. Jongbloed SL, Kassianos AJ, McDonald KJ, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 2010;207:1247-60

• **This study identifies a human DC subset that corresponds to mouse CD8α$^+$ DC specified for cross-presentation.**

20. Salaun B, Coste I, Rissoan MC, et al. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006;176:4894-901

• **The paper shows direct evidence that TLR3 signal is involved in triggering apoptosis in breast cancer cells.**

21. Nakamura M, Funami K, Komori A, et al. Increased expression of Toll-like receptor 3 in ntrahepatic biliary epithelial cells at sites of ductular reaction in diseasedlivers. Hepatol Int 2008;2:222-30

22. Choe J, Kelker MS, Wilson IA. Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science 2005;309:581-5

23. Liu L, Botos I, Wang Y, et al. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science 2008;320:379-81

24. Jelinek I, Leonard JN, Price GE, et al. TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. J Immunol 2011;186:2422-9

• **This paper shows that the length of dsRNA is a critical factor as a ligand for TLR3.**

25. Akira S. Toll-like receptor signaling. J Biol Chem 2003;278:38105-8

26. Funami K, Sasai M, Ohba Y, et al. Spatiotemporal mobilization of Toll/IL-1 receptor domain-containing adaptor molecule-1 in response to dsRNA. J Immunol 2007;179:6867-72

27. Funami K, Sasai M, Oshiumi H, et al. Homo-oligomerization is essential for Toll/interleukin-1 receptor domain-containing adaptor molecule-1-mediated NF-kappaB and interferon regulatory factor-3 activation. J Biol Chem 2008;283:18283-91

28. Salaun B, Coste I, Rissoan MC, et al. TLR3 can directly trigger apoptosis in

human cancer cells. J Immunol 2006;176:4894-901

29. McComb S, Cheung HH, Korneluk RG, et al. cIAP1 and cIAP2 limit macrophage necroptosis by inhibiting Rip1 and Rip3 activation. Cell Death Differ 2012;19(11):1791-801

30. Conforti R, Ma Y, Morel Y, et al. Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. Cancer Res 2010;70:490-500

31. Salaun B, Zitvogel L, Asselin-Paturel C, et al. TLR3 as a biomarker for the therapeutic efficacy of double-stranded RNA in breast cancer. Cancer Res 2011;71:1607-14

32. Akazawa T, Ebihara T, Okuno M, et al. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci USA 2007;104:252-7

• **The paper describes the participation of the TICAM-1 pathway in DC-driven NK activation.**

33. Lucas M, Schachterle W, Oberle K, et al. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity 2007;26:503-17

34. Azuma M, Ebihara T, Oshiumi H, et al. Cross-priming for antitumor CTL induced by soluble Ag + polyI:C depends on the TICAM-1 pathway in mouse CD11c(+)/CD8alpha(+) dendritic cells. Oncoimmunology 2012;1:581-92

• **The paper describes the participation of the TICAM-1 pathway in DC-driven CTL proliferation.**

35. Baron S, Bogomolova NN, Billiau A, et al. Induction of interferon by preparations of synthetic single-stranded RNA. Proc Natl Acad Sci USA 1969;64:67-74

36. Available from: http://www.innate-pharma.com/sites/default/files/uploads/IPH_3102_Nonconfidential_Summary_Feb10.pdf

37. Bernard JJ, Cowing-Zitron C, Nakatsuji T, et al. Ultraviolet radiation damages self noncoding RNA and is detected by TLR3. Nat Med 2012;18:1286-90

•• **An important study showing that a self noncoding RNA with stem-loop structures serves as a TLR3 ligand.**

38. Tatematsu M, Nishikawa F, Seya T, Matsumoto M. TLR3 recognizes single-stranded RNA with incomplete stem structures. Nat Commun 2013; submitted

• **This study highlights the viral RNAs with stems are sensed by TLR3 in a structure-dependent manner.**

39. Navabi H, Jasani B, Reece A, et al. A clinical grade poly I:C-analogue (Ampligen) promotes optimal DC maturation and Th1-type T cell responses of healthy donors and cancer patients in vitro. Vaccine 2009;27:107-15

40. Levy HB, Baer G, Baron S, et al. A modified polyriboinosinicpolyribocytidylic acid complex that induces interferon in primates. J Infect Dis 1975;132:434-9

41. Stephen EL, Hilmas DE, Mangiafico JA, Levy HB. Swine influenza virus vaccine: potentiation of antibody responses in rhesus monkeys. Science 1977;197:1289-90

42. Levy HB, Lvovsky E. Topical treatment of vaccinia virus infection with an interferon inducer in rabbits. J Infect Dis 1978;137:78-81

43. Olsen GA, Kern ER, Overall JC Jr. Effect of treatment with exogenous interferon, polyriboinosinic-polyribocytidylic acid, or polyriboinosinic-polyribocytidylic acid poly-L-lysine complex on Herpesvirus hominis infections in mice. J Infect Dis 1978;137:428-36

44. Machida H, Takezawa J, Kuninaka A, et al. Interferon induction and therapy of brain tumors in rats by poly(ICLC). Microbiol Immunol 1982;26:353-8

45. Galluzzi L, Vacchelli E, Eggemont A, et al. Trial watch: experimental toll-like receptor agonists for cancer therapy. Oncoimmunology 2012;1:699-716

• **A current review on clinical trials of TLR3 agonists with therapeutic potential.**

46. Braun W, Nakano M. Antibody formation: stimulation by polyadenylic and polycytidylic acids. Science 1967;157:819-21

47. Sugiyama T, Hoshino K, Saito M, et al. Immunoadjuvant effects of polyadenylic: polyuridylic acids through TLR3 and TLR7. Int Immunol 2008;20:1-9

48. Perrot I, Deauvieau F, Massacrier C, et al. TLR3 and Rig-like receptor on myeloid dendritic cells and Rig-like receptor on human NK cells are both mandatory for production of IFN-gamma in response to double-stranded RNA. J Immunol 2010;185:2080-8

49. Bot A, Smith D, Phillips B, et al. Immunologic control of tumors by in vivo Fc gamma receptor-targeted antigen loading in conjunction with double-stranded RNA-mediated immune modulation. J Immunol 2006;176:1363-74

50. Huang B, Zhao J, Li H, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. Cancer Res 2005;65:5009-14

51. Morikawa T, Sugiyama A, Kume H, et al. Identification of Toll-like receptor 3 as a potential therapeutic target in clear cell renal cell carcinoma. Clin Cancer Res 2007;13:5703-9

52. Kleinman ME, Yamada K, Takeda A, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 2008;452:591-7

53. Yu L, Chen S. Toll-like receptors expressed in tumor cells: targets for therapy. Cancer Immunol Immunother 2008;57:1271-8

54. Baron S, Bogomolova NN, Billiau A, et al. Induction of interferon by preparations of synthetic single-stranded RNA. Proc Natl Acad Sci USA 1969;64:67-74

55. Seya T, Matsumoto M. The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. Cancer Immunol Immunother 2009;58:1175-84

56. Yu M, Levine SJ. Toll-like receptor, RIG-I-like receptors and the NLRP3 inflammasome: key modulators of innate immune responses to double-stranded RNA viruses. Cytokine Growth Factor Rev 2011;22:63-72

57. Ebihara T, Azuma M, Oshiumi H, et al. Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. J Exp Med 2010;207:2675-87

58. Kumar H, Koyama S, Ishii KJ, et al. Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and

cytotoxic T cell responses. J Immunol 2008;180:683-7

59. McCartney S, Vermi W, Gilfillan S, et al. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J Exp Med 2009;206:2967-76

- **This paper clearly shows that polyI:C activates both TLR3 and MDA5 in vivo to induce NK cell activation.**

60. Miyake T, Kumagai Y, Kato H, et al. Poly I:C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways. J Immunol 2009;183:2522-8

61. Wu CY, Yang HY, Monie A, et al. Intraperitoneal administration of poly(I:C) with polyethylenimine leads to significant antitumor immunity against murine ovarian tumors. Cancer Immunol Immunother 2011;60:1085-96

62. Caskey M, Lefebvre F, Filali-Mouhim A, et al. Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. J Exp Med 2011;208:2357-66

- **The paper describes what happens in human volunteers injected with 1.6 mg polyI:CLC.**

63. Kato H, Takeuchi O, Mikamo-Satoh E, et al. Length-dependent recognition of doublestranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiationassociated gene 5. J Exp Med 2008;205:1601-10

64. Okada H, Kalinski P, Ueda R, et al. Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with {alpha}-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. J Clin Oncol 2011;29:330-6

- **The paper describes CTL response is induced in glioma patients by administration of tumor Ag peptides and polyI:CLC.**

65. Honda K, Taniguchi T. Toll-like receptor signaling and IRF transcription factors. IUBMB Life 2006;58:290-5

66. Iwakiri D, Zhou L, Samanta M, et al. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med 2009;206:2091-9

67. Samanta M, Takada K. Modulation of innate immunity system by Epstein-Barr virus-encoded non-coding RNA and oncogenesis. Cancer Sci 2010;101:29-35

68. Bortoluci KR, Medzhitov R. Control of infection by pyroptosis and autophagy: role of TLR and NLR. Cell Mol Life Sci 2010;67:1643-51

69. Lauterbach H, Bathke B, Gilles S, et al. Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 2010;207:2703-17

70. Thomas E, Gonzalez VD, Li Q, et al. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. Gastroenterology 2012;142:978-88

71. Kariko K, Ni H, Capodici J, Lamphier M. Weissman D. mRNA is an endogenous ligand for toll-like receptor 3. J Biol Chem 2004;279:12542-50

72. Swaminathan G, Rossi F, Sierra LJ, et al. A Role for microRNA-155 modulation in the anti-HIV-1 effects of toll-like receptor 3 stimulation in macrophages. PLoS Pathog 2012;8:e1002937

73. Proost P, Verpoest S, Van de Borne K, et al. Synergistic induction of CXCL9 and CXCL11 by toll-like receptor ligands and interferon-gamma in fibroblasts correlates with elevated levels of CXCR3 ligands in septic arthritis synovial fluids. J Leukoc Biol 2004;75:777-84

74. Vandenabeele P, Declercq W, Van Herreweghe F, Vanden Berghe T. The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. Sci Signal 2010;3:re4

75. Hitomi J, Christofferson DE, Ng A, et al. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 2008;135:1311-23

76. He S, Wang L, Miao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 2009;137:1100-11

77. Seya T, Shime H, Takaki H, et al. TLR3/TICAM-1 signaling in RIP3 tumor necroptosis. Oncoimmunology 2012;1:917-23

78. Eksioglu EA, Zhu H, Bayouth L, et al. Characterization of HCV interactions with Toll-like receptors and RIG-I in liver cells. PLoS One 2011;6:e21186

79. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526-9

80. Ebihara T, Shingai M, Matsumoto M, et al. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. Hepatology 2008;48:48-58

81. Lion E, Anguille S, Berneman ZN, et al. Poly(I:C) enhances the susceptibility of leukemic cells to NK cell cytotoxicity and phagocytosis by DC. PLoS ONE 2011;6:e20952

82. Ebihara T, Masuda H, Akazawa T, et al. Induction of NKG2D ligands on human dendritic cells by TLR ligand stimulation and RNA virus infection. Int Immunol 2007;19:1145-55

83. Zhou Z, Zhang C, Zhang J, Tian Z. Macrophages help NK cells to attack tumor cells by stimulatory NKG2D ligand but protect themselves from NK killing by inhibitory ligand Qa-1. PLoS ONE 2012;7:e36928

84. Ngoi SM, St Rose MC, Menoret AM, et al. Presensitizing with a toll-like receptor 3 ligand impairs CD8 T-cell effector differentiation and IL-33 responsiveness. Proc Natl Acad Sci USA 2012;109:10486-91

85. Ashkenazi A, Dixit VM. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 1999;11:255-60

86. Andersson A, Yang SC, Huang M, et al. IL-7 promotes CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer. J Immunol 2009;182:6951-8

87. Sawa Y, Arima Y, Ogura H, et al. Hepatic interleukin-7 expression regulates T cell responses. Immunity 2009;30:447-57

88. Starkhammar M, Kumlien Georen S, Swedin L, et al. Intranasal administration of poly(I:C) and LPS in BALB/c mice induces airway hyperresponsiveness and inflammation via different pathways. PLoS One 2012;7:e32110

89. Nagpal ML, Davis J, Lin T. Overexpression of CXCL10 in human prostate LNCaP cells activates its receptor (CXCR3) expression and inhibits cell proliferation. Biochim Biophys Acta 2006;1762:811-18

90. Kalinski P, Mailliard RB, Giermasz A, et al. Natural killer-dendritic cell

cross-talk in cancer immunotherapy. Expert Opin Biol Ther 2005;5:1303-15

91. Longhi MP, Trumpfheller C, Idoyaga J, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med 2009;206:1589-602

92. Purwar R, Schlapbach C, Xiao S, et al. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. Nat Med 2012;18:1248-53

93. Fujii S, Liu K, Smith C, et al. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med 2004;199:1607-18

94. Servet C, Zitvogel L, Hosmalin A. Dendritic cells in innate immune responses against HIV. Curr Mol Med 2002;2:739-56

Affiliation

Tsukasa Seya[†], Masahiro Azuma & Misako Matsumoto
[†]Author for correspondence
Hokkaido University,
Graduate School of Medicine,
Department of Microbiology and Immunology,
Kita-ku, Sapporo, 060-8638, Japan
Tel: +81 11 706 5073;
Fax: +81 11 706 7866;
E-mail: seya-tu@pop.med.hokudai.ac.jp

Arch. Immunol. Ther. Exp.
DOI 10.1007/s00005-012-0214-x

REVIEW

Multi-Step Regulation of Interferon Induction by Hepatitis C Virus

Hiroyuki Oshiumi · Kenji Funami · Hussein H. Aly · Misako Matsumoto · Tsukasa Seya

Received: 30 June 2012 / Accepted: 20 December 2012

Abstract Acute hepatitis C virus (HCV) infection evokes several distinct innate immune responses in host, but the virus usually propagates by circumventing these responses. Although a replication intermediate double-stranded RNA is produced in infected cells, type I interferon (IFN) induction and immediate cell death are largely blocked in infected cells. In vitro studies suggested that type I and III IFNs are mainly produced in HCV-infected hepatocytes if the MAVS pathway is functional, and dysfunction of this pathway may lead to cellular permissiveness to HCV replication and production. Cellular immunity, including natural killer cell activation and antigen-specific CD8 T-cell proliferation, occurs following innate immune activation in response to HCV, but is often ineffective for eradication of HCV. Constitutive dsRNA stimulation differs in output from type I IFN therapy, which has been an authentic therapy for patients with HCV. Host innate immune responses to HCV RNA/proteins may be associated with progressive hepatic fibrosis and carcinogenesis once persistent HCV infection is established in opposition to the IFN system. Hence, innate RNA sensing exerts pivotal functions against HCV genome replication and host pathogenesis through modulation of the IFN system. Molecules participating in the RIG-I and Toll-like receptor 3 pathways are the main targets for HCV, disabling the anti-viral functions of these IFN-inducing molecules. We discuss the mechanisms that abolish type I and type III IFN production in HCV-infected cells, which may contribute to understanding the mechanism of virus persistence and resistance to the IFN therapy.

Keywords Hepatitis C virus · TLR3 · TICAM-1 (TRIF) · MAVS (IPS-1, Cardif, VISA) · Interferon-inducing pathway · Double-stranded RNA

Abbreviations

BMDC	Bone marrow-derived dendritic cells
CTL	Cytotoxic T lymphocytes
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dsRNA	Double-stranded RNA
IFN	Interferon
LD	Lipid droplet
MAM	Mitochondrial-associated endoplasmic reticulum membranes
MAVS	Mitochondrial antiviral signaling protein
Mf	Macrophages
mRNA	Messenger RNA
NK	Natural killer
NS	Non-structural
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
STING	Stimulator of IFN genes
TICAM-1	Toll-IL-1-homology domain-containing adaptor molecule-1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	TNF-α receptor 1

MAVS has been identified as the adaptor for RIG-I and MDA5 by four independent groups, and then also known as IPS-1, Cardif or VISA (Kawai and Akira 2009). TICAM-1 has been identified as the adaptor for TLR3 and TLR4 by two independent groups, and thus also described as TRIF (Oshiumi et al. 2003). In accordance with the HUGO Gene Nomenclature Committee-approved nomenclature, here we refer to these adaptor molecules as MAVS and TICAM-1, respectively.

H. Oshiumi · K. Funami · H. H. Aly · M. Matsumoto · T. Seya (✉)
Department of Microbiology and Immunology,
Hokkaido University Graduate School of Medicine,
Kita, Sapporo 060-8638, Japan
e-mail: seya-tu@pop.med.hokudai.ac.jp

Published online: 05 January 2013

Introduction

Hepatitis C virus (HCV) mainly infects human hepatocytes, and triggers induction of cytokines and type I (IFN-α/β) and type III interferons (IFN-λ) (Fig. 1). Although cells expressing IFN receptors respond to the released IFN and amplify type I IFN production, IFN induction is not always robust in the infected cells due to the fact that HCV proteins inhibit host IFN-inducing pathways. IFN-stimulated genes (ISGs), such as IRF-7, MAP3K14, RIG-I, IRF-2, and IRF-1 are known to inhibit HCV replication (Schoggins et al. 2011). In particular, type III IFNs are more produced than IFN-α/β in HCV-infected hepatocytes via the mitochondrial antiviral signaling protein (MAVS) pathway to induce a set of ISGs (Thomas et al. 2012). Cytokines and chemokines are released from infected hepatocytes and myeloid cells in the liver. These mediators affect the formation of inflammatory environments and modify homeostasis of the host cell community, including the recruited bystander cells. Although these scenarios generally reflect the signs of patients with HCV, what occurs following initial virus entry into host cells remains obscure at the molecular level. HCV genome RNA is internalized via fusion and a portion of 3′-polyU/UC or 5′-triphosphate-short stem RNA acts directly as a ligand for RIG-I (Saito et al. 2008). The HCV genome functions as a messenger (m)RNA for HCV polyprotein production and, at the same time, HCV genome replicates in the cytoplasm (Lindenbach et al. 2007). Double-stranded (ds)RNA accumulating in infected cells is the main pattern molecule (PAMP) and, once liberated, provokes activation of innate immunity in myeloid cells. How host cells sense HCV RNA or dsRNA during infection and replicon transfection has been investigated, and has led to an understanding of the importance of the cytoplasmic RNA recognition pathways (Fig. 1), particularly, the MAVS pathway (Cheng et al. 2006; Li et al. 2005a, b). The current concept is that MAVS signals the kinases, TANK-binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε), to phosphorylate IFN regulatory factors (IRF)-3 and IRF-7, resulting in the induction of type I IFN (Kawai and Akira 2009). Likewise, IRF-3 and nuclear factor (NF)-κB appear to participate in

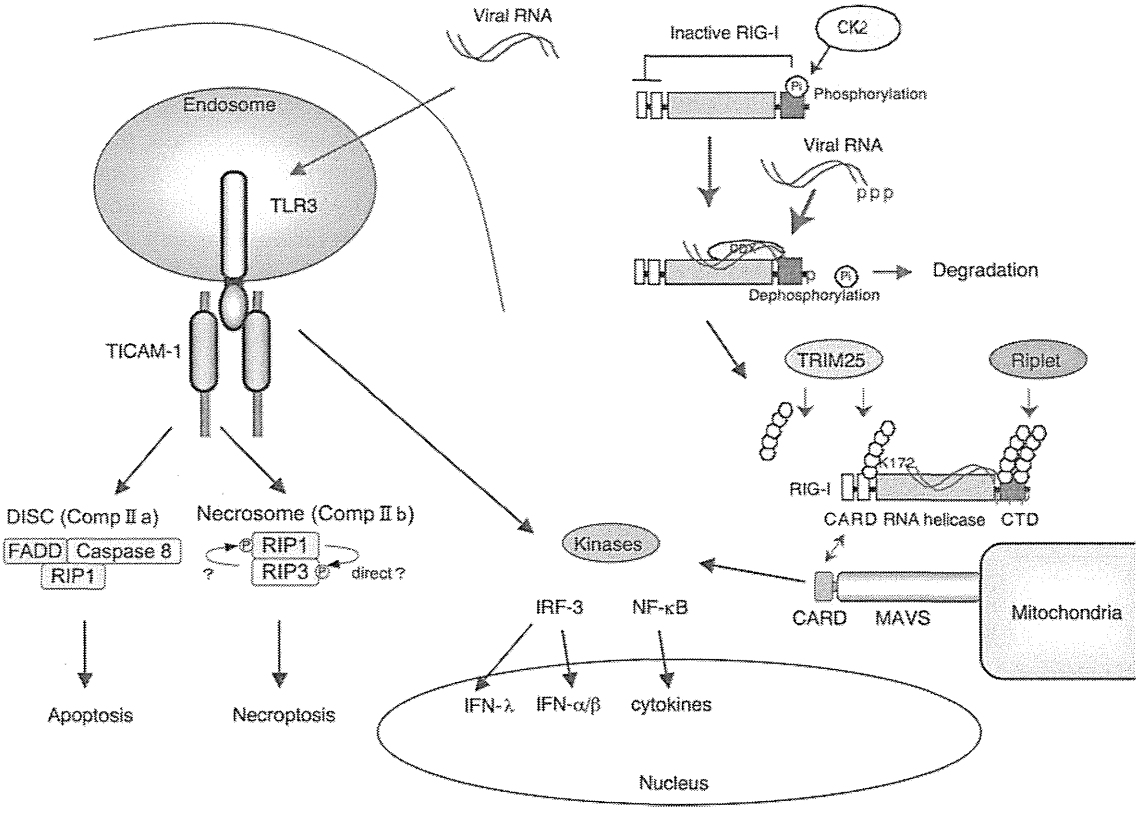

Fig. 1 Cytoplasmic and endosomal sensors for virus dsRNA in HCV infection. Live signal (*right*) and cell death signal (*left*) in response to viral dsRNA are illustrated. The live signal occurs with stimulation of TLR3 or RIG-I-like receptors and essentially induces activation of NF-κB to support induction of pro-inflammatory cytokines and type I/type III interferons (IFNs) (*right*). This live signal may be amplified by the function of a small amount of IFN-β that is required for the maintenance of homeostasis of the cellular microenvironment. In contrast, death signal occurs with TLR3: caspase 8 is a key molecule for discriminating between apoptosis and necroptosis, and its functional absence sustains the RIP1/RIP3 necrosome signal leading to necroptosis (*left*). Viral dsRNA recognition by RIG-I is induced by RIG-I ubiquitination (*right*). The two modes of K63 polyubiquitination activate RIG-I

the induction of IFN-λ (Ding et al. 2012). Because RIG-I-like receptors are IFN-inducible genes, only trace levels are found in resting cells or those in the early stage of virus entry. Thus, how RIG-I/MDA5 captures internalized or replicating virus RNA to evoke an antiviral response in such situations remains unexplained.

There are many reports suggesting that Toll-like receptor 3 (TLR3) participates in the response to HCV dsRNA (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012) (Fig. 1). Most of the relevant studies have been performed with hepatoma cell lines due to the lack of proper systems for reproducing the HCV life cycle in culture as well as the in vivo animal model to examine the HCV immune responses. Cell death accompanied with a cytopathic effect is another phenotype of infected hepatocytes (Lim et al. 2012). Hepatocyte death is characterized as apoptosis, but the possible involvement of the pathway with receptor-interacting protein (RIP) kinases in infection-induced cell death has not been strictly ruled out (Fig. 1). Necrosis-like cell death (necroptosis) might cause a source of infectious virions and lead to the pathogenesis of HCV-associated liver damage. Ligands of the death receptor family, including FasL and TRAIL, are likely to associate with hepatocyte death induced by HCV infection (Bantel and Schulze-Osthoff 2003; Saeed et al. 2011; Zhu et al. 2007); however, what triggers the induction of the effector cells is still undetermined. Apart from these cell death family proteins, it is accepted that TLR3 is an activator of the RIP1 pathway (Meylan et al. 2004), which clearly participates in macrophage necroptosis (He et al. 2011). TLR3 is up-regulated in macrophages/dendritic cells (Mf/DC) in an IFN-dependent manner (Tanabe et al. 2003) and recognizes internalized virus dsRNA in the endosome of these phagocytes (Matsumoto et al. 2011). TLR3 has been characterized as an inducer of cellular immune effectors (Matsumoto et al. 2011; Seya and Matsumoto 2009). In accordance with the current dogma, natural killer (NK)-ligand up-regulation or cross-presentation of DCs that occurs with the internalization of dead cell-derived dsRNA may bridge the missing link between HCV dsRNA and TLR3-derived DC maturation (Ebihara et al. 2008).

Dead cells are a source of damage-associated molecular pattern (DAMP) (Kono and Rock 2008). DAMP refers to an intracellular molecule with inflammation-inducing capacities when it is released out of the cell. DAMP does not belong to the cytokine family, but resembles PAMP in its functional properties toward activation of myeloid DCs and macrophages (Kono and Rock 2008). Its function may be associated with physiological responses related to HCV immune response in a broad sense, including regeneration and tumorigenesis. Recently, necrotic or necroptotic cell death has been closely connected with innate immune responses involving pattern sensing (Kono and Rock 2008; Nace et al. 2012).

Table 1 Sensors for nucleic acid PAMPs and DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids (PAMP)	
Cytosolic long dsRNA	MDA5
Cytosolic 5′-PPP-RNA	RIG-I
Endosomal >140 bp dsRNA	TLR3
Nonmethylated CpG DNA	TLR9
Cytosolic dsDNA	DNA sensors[a]
Self-molecular patterns (DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4[b]
S100 proteins	RAGE
Self-nucleic acids (DAMP)	
Self-DNA	DNA sensors[a]
Self-mRNA	TLR3

HMGB1 High-mobility group box 1, *HSPs* heat shock proteins

[a] See Table 2

[b] Scavenger receptors, CD91, etc.

How HCV patterns are sensed and linked to the cellular immunity will be an intriguing issue. DAMP contains a number of cytosolic or nucleic molecules, as in Table 1, and in particular nucleic acids from infected cells. Thus, DAMP and dsRNA of viral origin are extrinsic patterns for sensors to evoke unique features of inflammation during HCV infection.

Herein, we discuss the interrelationship between these recent findings on innate immunity and HCV infection.

Blocking IFN Induction by HCV Proteins

Proteolytic Control of the IFN-Inducing Pathways by NS3/4A

HCV genome RNA serves as a single mRNA that encodes ~3,000 amino acids, consisting of 10 virus proteins (Lindenbach et al. 2007). Structural proteins (core, E1, and E2) are situated at the N-terminal region of this polyprotein (Fig. 2a). The HCV polyprotein is first cleaved between 191A and 192Y by signal peptidase to separate the core protein from E1 protein and the core is retained on the endoplasmic reticulum (ER) membrane (McLauchlan et al. 2002). Then, signal peptide peptidase scissors out the core protein by cleavage at 177F and 178L from the ER membrane (Okamoto et al. 2004). E1 and E2 are also released from the remaining structural protein complex by the proteolytic function of signal peptidase (McLauchlan et al. 2002). Non-structural proteins of HCV are fragmented into functional units by NS2 and NS3/4A proteases. Hence, the release of the structural proteins precedes the mature

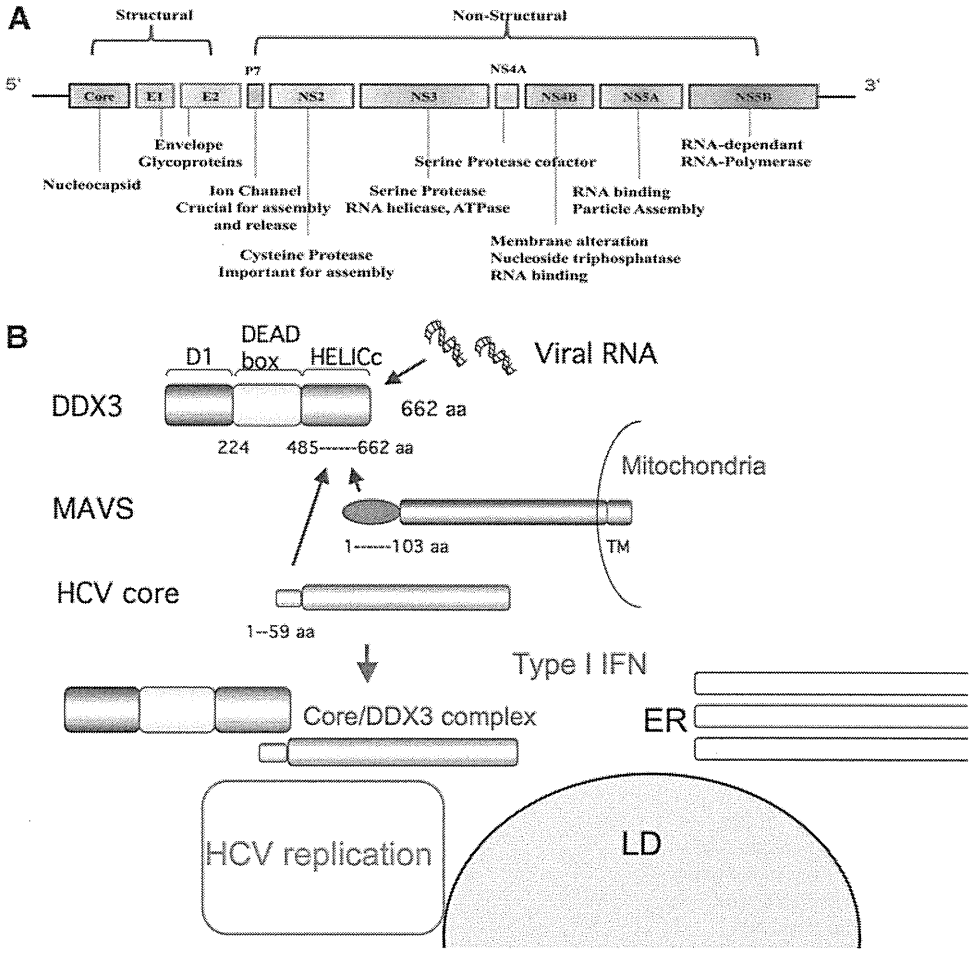

Fig. 2 Two different functions of HCV core protein. HCV genome and the functions of each HCV protein. HCV core is first clipped out from the polyprotein of HCV, and later NS proteins are generated (**a**). HCV core protein retracts DDX3 from the MAVS–DDX3 signal complex on the mitochondria (**b**). DDX3 usually couples with MAVS on mitochondria and directly binds overwhelmed virus dsRNA in virus-infected cells. When the HCV core protein is produced, DDX3 binds core protein with high affinity and moves from the mitochondria to the HCV replication apparatus, where the core is recruited. The HCV replication apparatus is situated near the lipid droplet (LD) in ER. DDX3 supports HCV replication in the apparatus

processing of non-structural (NS) proteins during the HCV polyprotein processing (Lindenbach et al. 2007). Notably, two structural proteins, core and E2, exhibit regulatory functions against type I IFN induction (Florentin et al. 2012; Mulhern and Bowie 2010).

NS3/4A protease is reported to be crucial, not only for the liberation of HCV NS proteins, but also for the regulation of host anti-viral reactions by proteolytic inactivation of host cytosolic proteins, which also interfere with homeostasis of live cells. It has been reported that NS3/4A proteolytically degrades MAVS (Cheng et al. 2006; Li et al. 2005b; Loo et al. 2006). In addition, NS4B protein has been reported to target STING to repress RIG-I-mediated type I IFN induction in hepatocytes (Nitta et al. 2012). Preceding the generation of these NS proteins, HCV core (Oshiumi et al. 2010a) and E2 proteins (Florentin et al. 2012) can suppress RIG-I-mediated type I IFN production in hepatocytes and plasmacytoid DC, respectively. In particular, the generation of core protein and NS3/4A are closely associated with suppression of the HCV-mediated host IFN biological response and the promotion of HCV replication. Since core protein is produced prior to NS3/4A in HCV-infected cells, many other functions of the core are expressed just before the proteolytic processing of HCV NS proteins within the cells.

NS3/4A cleaves MAVS (Cheng et al. 2006; Li et al. 2005b; Oshiumi et al. 2010a) and TICAM-1 (Li et al. 2005a). Hence, NS3/4A proteolytically controls at least two adaptor proteins as its substrates. In addition, Riplet is reduced in response to HCV replication (Oshiumi et al. 2010c). Whether or not Riplet is a substrate for NS3/4A is still under investigation.

MAVS Inactivation by NS3/4A

The current assumption is that the RIG-I family proteins, RIG-I and MDA5, sense viral RNA to induce type I IFN and pro-inflammatory cytokines, which in turn suppress viral infection. RIG-I and MDA5 possess the N-terminal caspase activation and recruitment domain (CARD), the central DExD/H-box helicase domain, and the C-terminal RNA-binding domain (CTD) (Fig. 1). According to crystal structure analysis, the basic region of CTD binds virus dsRNA irrespective of the presence of 5′-triphosphate (Yoneyama et al. 2004), while the CARD domain participates in interaction with the adaptor.

RIG-I recognizes HCV RNA, as well as its replication intermediate, dsRNA (Saito et al. 2008). Although MDA5 recognizes long dsRNA patterns, the role of MDA5 in HCV RNA recognition is unknown. MAVS is the key adaptor for RIG-I/MDA5-mediated IFN induction in HCV infection, although it is localized in the mitochondrial outer membrane apart from intact RIG-I molecules (Seth et al. 2005). RIG-I is not quantitatively sufficient at the protein level to capture the abundant dsRNA replicating in infected cells during the early stage; other molecules have to accept the overwhelmed dsRNA in other cytoplasmic regions (Oshiumi et al. 2010b). RIG-I is initially involved in a molecular complex (RIG-I granule), which contains many other molecules that make up a nucleocapture complex. E3 ubiquitin ligases are involved in the RIG-I granule. Ubiquitin ligases, TRIM25 (Gack et al. 2007) and Riplet (Oshiumi et al. 2009), are also situated in the RIG-I granule together with RIG-I and confer RNA-binding capacity on RIG-I through RIG-I ubiquitination (Fig. 1). TRIM25 ubiquitinates N-terminal lysines of RIG-I (Gack et al. 2007), while Riplet ubiquitinates C-terminal lysines of RIG-I (Oshiumi et al. 2009), either or both of these molecules enable RIG-I to interact with MAVS and confer mobility on mitochondria (Fig. 3). A recent report

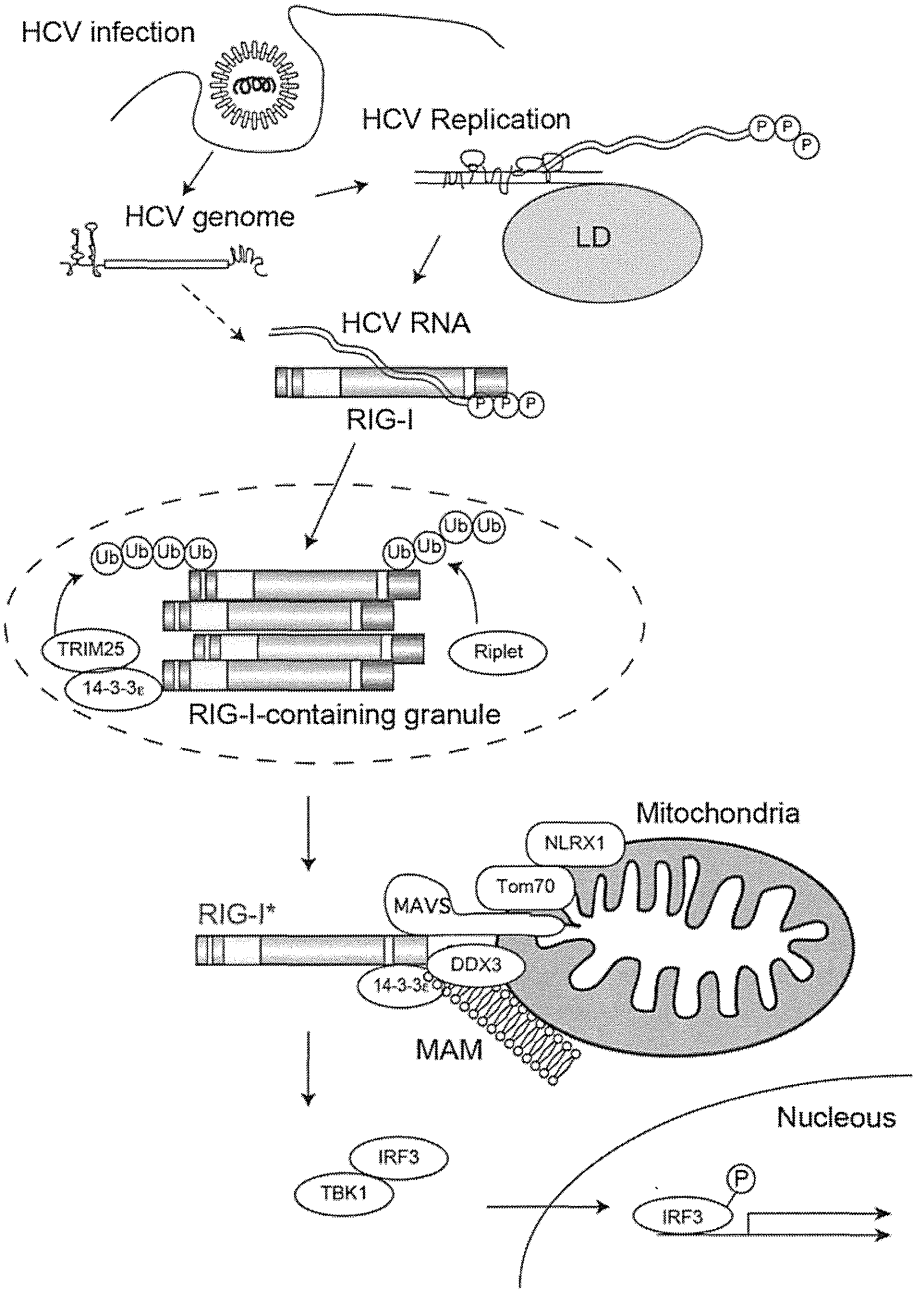

Fig. 3 Translocation of RIG-I from the cytoplasm to the mitochondria. RIG-I is diffusely distributed in the cytoplasm. When minute quantities of dsRNA enter the cytoplasm (*dashed line*), the RIG-I granule is formed with many other molecules to sense dsRNA. Once RIG-I molecules are polyubiquitinated, they form a complex with dsRNA and becomes mobile (RIG-I*). RIG-I* is recruited to the mitochondria to couple with MAVS. There are many other molecules associated with mitochondrial signaling. Because DDX3 captures overwhelmed dsRNA, the RIG-I–DDX3–MAVS complex allows robust IFN production in conjunction with dsRNA/DDX3. Whether DDX3 participates in IFN-λ induction remains undetermined

speculated that after RIG-I is up-regulated and ubiquitinated in the RIG-I granule, the 14-3-3ε is coupled with newly ubiquitinated RIG-I (Liu et al. 2012). Then, RIG-I moves from the granule to the mitochondrial membrane, a distinct membrane compartment linked to the ER, which is referred to as mitochondrial-associated endoplasmic reticulum membranes (MAM) (Horner et al. 2011). MAM accumulates MAVS and may coordinate MAVS signaling of innate immunity from peroxisomes (Dixit et al. 2010) and mitochondria (Seth et al. 2005), while MAVS localized to MAM serves as a molecular platform for the IFN-inducing signal. MAVS is constitutively complexed with DDX3, which serves as an acceptor of dsRNA in resting (RIG-I-insufficient) cells (Oshiumi et al. 2010b). If this is the case, the location for RIG-I ubiquitination (i.e., RIG-I granule) may differ from the site at which RIG-I interacts with the MAVS–DDX3 complex for signaling. Validating this issue will be of great interest in understanding initial viral RNA recognition.

Our previous data suggested that three forms of MAVS are detected in HCV-replicating hepatocyte lines by imaging analysis, as follows: intact MAVS, sequestered MAVS, and proteolytically liberated MAVS (Oshiumi et al. 2010a). These forms of MAVS simultaneously exist in hepatocytes expressing the HCV replicon or those infected with HCV. The MAVS proteolytically released from mitochondria appear to have decreased ability to activate IRF-3. MAVS is also diminished in some HCV-infected cells to lose its IFN-inducing function (Oshiumi et al. 2010a), suggesting that NS3/4A is a protease that determines the inactivation state of MAVS in HCV-replicating hepatocytes. A recent report suggested that Riplet is depleted during HCV replication (Oshiumi et al. 2010c), indicating the possibility that participation of the expressed NS3/4A protease in degrading other molecules upstream of MAVS is more important for IFN regulation than clipping out of MAVS in infected cells (Fig. 1). Similarly, other factors independent of proteolytic control may be critical for dsRNA-mediated IFN inducibility, as demonstrated by Cheng et al. (2006).

Blocking of the DDX3-Augmented IFN Production by Core Protein

Three reports have independently showed that DEAD/H Box 3 (DDX3, also known as DBX) acts as a positive regulator for MAVS-mediated type I IFN induction (Table 2; Fig. 2b). Elevation of MAVS pathway-mediated type I IFN production by DDX3 is modally different in these three reports (Mulhern and Bowie 2010). Like RIG-I and MDA5, DDX3 is a member of the DExD/H-box family of RNA helicases and is ubiquitously expressed in a variety of cells (Kim et al. 2001). The DExD/H motif of the members in this family of proteins is predictive of a role in RNA-binding and RNA-dependent cellular processing (Schroder 2009). Schroder et al. (2008) showed that the vaccinia virus protein K7 binds DDX3 and inhibits pattern-recognition receptors-induced IFN-β promoter activation. They suggested that DDX3 interacts with IKKε to enhance IRF-3 activation, while K7 counters DDX3 activity of MAVS-mediated IFN-β induction. This IFN-enhancing function of DDX3 in IRF-3 activation is located at the N-terminus of DDX3, which is the same region of the protein targeted by K7 for IRF-3 inhibition. Structure analysis of K7 complexed with a peptide from the N-terminus of DDX3 (Oda et al. 2009) has confirmed this finding.

Table 2 Nucleic acid sensors related to IFN induction in innate immunity

Pattern-recognition receptors	Adaptors	Agonists (references)	Origin
MDA5	MAVS	Cytosolic long dsRNA (Yoneyama et al. 2008)	RNA viruses
RIG-I	MAVS	Cytosolic 5′-PPP-RNA (Yoneyama et al. 2008)	RNA viruses
NOD2	MAVS	Cytosolic ssRNA (Morosky et al. 2011)	RNA viruses
TLR3	TICAM-1	Endosomal >140 bp dsRNA viruses, host (Jelinek et al. 2011)	DNA/RNA viruses
TLR7/8	MyD88	Endosomal ssRNA (Uematsu and Akira 2007)	RNA viruses, bacteria
TLR9	MyD88	Nonmethylated CpG DNA (Uematsu and Akira 2007)	RNA viruses, bacteria
DDX3	MAVS	dsRNA, ssRNA (Oshiumi et al. 2013; this review)	Viruses, host
DDX1/21, DHX36	TICAM-1	dsRNA (Rathinam and Fitzgerald 2011)	Viruses?
DDX60	MAVS	dsRNA, ssRNA, dsDNA (Oshiumi et al. 2013; this review)	Viruses, host
DHX9/DHX36 MyD88	STING	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DDX41	TBK1	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DAI (ZBP1)	STING	dsDNA (Takaoka and Taniguchi 2008)	DNA viruses
IFI16	β-Catenin	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
LRRFIP1		dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses

ssRNA single-stranded RNA

The second study indicated that DDX3 constitutively interacts with MAVS via the C-terminal region of DDX3 (Oshiumi et al. 2010b). The binding of DDX3 to MAVS is constitutive and not through the N-terminus, in contrast to the case of the virus-dependent interaction between DDX3 and IKKε (Schroder et al. 2008). RIG-I-induced IFN-β promoter reporter gene activity is inhibited by DDX3 small interfering RNA and enhanced by overexpression of DDX3 (Oshiumi et al. 2010b). Thus, DDX3 synergistically activates the IFN-β promoter together with MAVS. The C- and N-terminal regions of the DDX3 regulate MAVS-mediated IFN induction (Hogbom et al. 2007) (Fig. 1).

In contrast, Soulat et al. (2008) demonstrated a positive role for DDX3 in IFN-β promoter induction in another distinct manner. Specifically, DDX3 is shown to be a kinase substrate for TBK1 to synergistically enhance IFN-β promoter activation by TBK1. Furthermore, they demonstrated that DDX3 is recruited to the IFN-β promoter via its N-terminal region (Soulat et al. 2008). Together, these findings show that DDX3 is a positive regulator targeting the multiple sites of the RLR-induced IFN pathway. A role for DDX3 in cell cycle control and apoptosis has also been proposed in response to dsRNA (Schroder et al. 2008).

DDX3 facilitates viral replication in a variety of viruses, including HCV. The N-terminus of HCV core protein binds the C-terminus of DDX3 (Owsianka and Patel 1999). According to a recent finding, the HCV core protein participates in the suppression of DDX3-augmented MAVS signaling for IFN-β induction (Fig. 2b), which may also be related to the function of DDX3 described in the second study mentioned before (Oshiumi et al. 2010b). Unlike the DExD/H-box helicases, such as RIG-I and MDA5, DDX3 is constitutively expressed and co-localized with MAVS around mitochondria (Table 2). However, HCV core protein interferes with DDX3 function to enhance MAVS signaling by coupling with DDX3 to dissociate it from MAVS in the mitochondria. In hepatocytes with the HCV replicon, DDX3/MAVS-enhanced IFN-β-induction is largely abrogated, even when DDX3 is co-expressed. Whether DDX3 enhances IFN-λ induction like RIG-I remain untested. DDX3 is spotted with minimal merging with MAVS in confocal analysis, and partly assembles in the HCV core protein located near the lipid droplet (LD) in replicon-positive hepatocytes, although in some cells MAVS is diminished or disseminated apart from mitochondria. Thus, HCV core retracts DDX3 from MAM, where RIG-I moves from the RIG-I granule to assemble together with MAVS (Figs. 2b, 3).

Our consensus is that the binding of HCV core protein to DDX3 and suppressing DDX3–MAVS complex formation are crucial for inhibition of the MAVS pathway. However, multiple functions of DDX3 may be reflected in other functional aspects of core protein; specifically, the DDX3–core interaction is required for HCV replication (Ariumi et al. 2007). Although DDX3 promotes efficient HCV infection by accelerating HCV RNA replication, the processes appear independent of its interaction with the viral core protein (Angus et al. 2010). In addition, the association between DDX3 and core protein implicates DDX3 in HCV-related hepatocellular carcinoma progression (Chang et al. 2006). Based on its core protein association and MAVS-regulatory properties, DDX3 appears to be switched by the core protein from an HCV-suppressing (i.e., IFN-inducing) mode to an HCV replication-supporting mode (Fig. 2b). The results enable us to conclude that HCV infection is promoted by modulating the dual function of DDX3.

Evidence is accumulating that HCV assesses many steps in the IFN-inducing pathway throughout the early and late infection stages, and suppresses IFN production by multiple means. Disruption of MAVS function by NS3/4A and core protein may be crucial in HCV-infected Huh7.5 cells, even though the cells harbor dysfunctional RIG-I (Binder et al. 2007). Type I IFN suppresses tumor progression by causing expression of p53 and other tumor-suppressing agents (Takaoka et al. 2005). E2 and NS4B may affect tumor progression by controlling type III IFN induction. These unique features of the HCV proteins require further confirmation and should be in the focus of investigation regarding HCV persistence, chronic infection, and progression to cirrhosis and carcinoma.

Inactivation of the TICAM-1 Pathway by NS3/4A

TICAM-1 pathway has been associated with chemokine production, apoptosis, necroptosis, and IL-12p40 production in hepatoma cell lines expressing TLR3 (Li et al. 2012); however, such immune responses are predominantly absent in primary cultured cells. This might be explained by the TLR3 signaling that is likely to be shut off in most normal hepatocytes, but executed in hepatocytes of the infectious liver during chronic states of HCV infection or exposure to dsRNA stimulation. Despite the constitutive expression of the adaptor molecule TICAM-1 in human hepatocytes, only trace amount of TLR3 is being expressed in comparison to RIG-I that is commonly expressed. This gives us an insight of the role played by other cytoplasmic dsRNA sensors such as DDX1/DDX21/DHX36 (Table 2), in the activation of TICAM-1 pathway (Zhang et al. 2011). It remains unknown whether these cytoplasmic dsRNA sensors participate in IFN-λ induction.

Although there are positive findings supporting the importance of TLR3 in the pathogenesis of HCV infection (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012), the expression level of TLR3 is still a contentious issue. TLR3 protein is undetectable by immunostaining with monoclonal antibody (TLR3.7) against huTLR3 in

uninfected human hepatocytes (Nakamura et al. 2008) or Huh-7 hepatoma cells that are commonly used for propagation of HCV (Wang et al. 2009). Only a few reports have shown evidence that TLR3 protein is weakly detected in resting primary cultured hepatocytes (Wang et al. 2009). On the other hand, several reports have suggested that the TLR3 at the messenger level was observed in cultured hepatocytes and hepatoma cell lines by real-time polymerase chain reaction (Khvalevsky et al. 2007). Since TLR3 expression is partly regulated by p53, mutated p53 in Huh-7 cells as well as other hepatocellular carcinoma cell lines may attribute to the specific lacking of the TLR3 expression. In addition, Tanabe et al. (2003) demonstrated that the lack of TLR3 expression in Huh-7 cells may be due to other transcriptional regulations.

TLR3 expression appears to be up-regulated in cultured hepatocytes in response to polyI:C (Wang et al. 2009). Similarly, TLR3 is up-regulated in hepatocytes of patients with chronic HCV infection or polyI:C-injected mice (McCartney et al. 2009; Nakamura et al. 2008). These results lead to the assumption that TLR3 can be up-regulated in hepatocytes in an infectious milieu in response to the produced dsRNA or liberated type I IFN by HCV-infected hepatocytes via the RIG-I-mediated IFN-inducing pathway. A similar mechanism of secondary induction of the TLR3 expression by type I IFN may as well occur in hepatoma cells. Thus, HCV infection or malignant transformation allows hepatocytes to turn TLR3 positive, enabling the activation of the TICAM-1 pathway by external virus dsRNA. This would explain the finding that TLR3 is highly expressed in biliary epithelial cells and certain hepatoma cell lines (Harada et al. 2007; Nakamura et al. 2008). Li et al. (2012) showed that TLR3 senses HCV infection and induces ISG expression when TLR3 is over-expressed artificially in the Huh7.5 cells that are deficient in RIG-I signaling. They demonstrated that HCV replication is partially restricted when the cells are infected at a low multiplicity. However, such protective effect is dismissive when the infection is overwhelmed at high multiplicity partly due to the limitation of the TLR3.

TLR3 has a distinct feature from RIG-I as TLR3 can potentially sense viral dsRNA released into the extracellular environment by other cells. Considering the fact that NS3/4A protease that interferes with the host anti-viral reactions is only expressed within infected cells, TLR3-mediated immune responses might be triggered in the uninfected hepatocytes or other cell types. On top of that, it has been reported that TICAM-1 is also a substrate for NS3/4A protease in hepatoma cell lines (Li et al. 2005a). Ferreon et al. (2005), has confirmed this in the corollary biochemical studies. TICAM-1 and its signal pathway are intact within the cells around infected hepatocytes (Shimoda et al. 2011). This may contribute to the IFN responses observed in some patients.

TLR3 is highly expressed in biliary epithelial cells where biliary atresia occurs in response to the interaction between TLR3-stimulated monocytes and liver NK cells (Harada et al. 2007; Shimoda et al. 2008, 2011). However, it remains intriguing whether the up-regulated TRAIL or FasL in NK cells resulted from the IFN-signaling (Estornes et al. 2012) are responsible for the induction of cell death in hepatocytes. Nevertheless, TICAM-1-mediated cell death in some HCV-infected hepatocytes is also likely to occur via autophagy or *trans*-acting of dsRNA generated by HCV replication.

DAMP and dsRNA in HCV Pathology

Live or death signals are usually raised by viral dsRNA in virus-infected cells. Type I/type III IFNs and proinflammatory cytokines (IL-6, IL-12, TNF-α etc.) are liberated through IRF-3/7 and NF-κB activation as the output from virus-infected cells that are alive. In contrast, DAMP and cytoplasmic cytokines converted to active forms by caspase 1 eventually result from activation of inflammasome and often links to cell death events (Yeretssian 2012). TLR, Nod-like receptor and other cytosolic nucleic acid sensors are closely associated with PAMP/DAMP recognition (Table 2), therefore inflammation states are fundamentally modified by these factors (Bortoluci and Medzhitov 2010).

Replication of virus RNA allows hepatocytes to induce type III IFN, IL-7 and chemokines (Apolinario et al. 2005; Zeremski et al. 2007). HCV also regulates production of chemokines (Sillanpaa et al. 2008) and type III IFNs (Thomas et al. 2012) by infected cells. Polymorphisms around the IL-28B gene have been associated with clearance of HCV in human, indicating a role for type III IFNs rather than type I in HCV infection (Thomas et al. 2012), although little is known about the function of type III IFNs in intrinsic antiviral responses. IFNs and IL-7 released from HCV-infected hepatocytes possibly act on myeloid cells and lymphocytes expressing their receptors to induce IFN-γ (Sawa et al. 2009). Once type I/type III IFN and IFN-γ are systemically distributed, synergistic function of these IFNs allows the systemic cells to produce ISGs including CXCL10 (IP-10) and CCL5 (RANTES) (Larrubia et al. 2008; Zeremski et al. 2007). In addition, dsRNA-induced IL-7 forms a positive amplifying loop with T-cell-derived IFN-γ to promote macrophage recruitment and CXCR3 ligand (CXCL10) expression by these macrophages (Andersson et al. 2009). Since CXCR3 is mainly expressed on activated T and NK cells, these cytotoxic effectors gather around the inflammatory nest as well as secondary affected organs. HCV-related extrahepatic

disorders are likely to occur in conjunction with ectopic T-cell immune response (Antonelli et al. 2008, 2009). In addition, these immunological aberrances may be modulated by viral factors. In fact, in mouse models, NS5A expression impairs clearance of other viruses from the liver due to the inhibition of IFN-γ production (Kanda et al. 2009). By any means, induction of IFN-γ in concert with activation of cellular immunity is a major array for live signal in HCV infection.

Necrosis-like cell death occurs contrarily in a cell type-specific manner as a result of death signal. Tumor necrosis factor (TNF)-α and its receptor, TNFR1, are implicated in this process. The coupling of RIP1 with RIP3, termed a necrosome, is responsible for the switching of apoptosis to necroptosis (Cho et al. 2009; He et al. 2009). Caspase 8 acts as a key protease for blocking the formation of the necrosome; the RIP1/RIP3 complex can assemble only in the absence of functional caspase 8. It has been reported that virus dsRNA often induces apoptosis in infected cells, which is known as cytopathic effect (Lim et al. 2012). TICAM-1 and RIP1 may be involved in the virus-derived apoptosis. Yet, possible involvement of RIG-I/MDA5 in cell death cannot be ruled out in some cases of viral infection (Eksioglu et al. 2011). In HCV-infected hepatocytes, how the TLR3/TICAM-1 pathway is involved in necroptotic inflammation is the next issue to be elucidated with respect to HCV pathogenesis (Fig. 1). HCV dsRNA and DAMP can be liberated from infectious hepatocytes, as well as virus particles. TNF-α and IL-6 are the pro-inflammatory cytokines released during HCV infection. Hence, a characteristic feature of the HCV-infected hepatocytes is that DAMP is released together with viral dsRNA from necroptotic HCV-infected cells to the surrounded environment. These factors stimulate nucleic acid sensors of myeloid DC/Mf in the draining region (Table 2). We expect that necroptosis will be of enormous interest in HCV infection following smoldering inflammation. Because each virus species harbors distinct strategies for escaping the innate dsRNA-sensing system, the physiological role of TLR3-mediated necroptosis should be analyzed in a HCV-specific fashion.

Cellular Immunity Induced by HCV-Infected Cell Debris

Once DC/Mf responds to these unusual innate stimulators, DAMP and dsRNA, cellular immunity is provoked against HCV Ag with irregular modification by these immune enhancers during HCV infection. NK cells and cytotoxic T lymphocytes (CTL) are known to be driven in myeloid DCs by stimulation with dsRNA, and in fact are the main effectors against HCV-infected hepatocytes based on several different systems (Ebihara et al. 2008; Saeed et al. 2011; Zhu et al. 2007). DCs express NK-activating ligands by recognizing dsRNA to activate NK cells (Ebihara et al. 2007), and cell damage is reported to play a role in the regulation of NK-activating ligands (Wen et al. 2008), thereby dsRNA and DAMP are involved in the elimination of HCV-infected cells. Subsequently, DCs cross-prime CD8 CTLs through incorporation of dsRNA and HCV Ag-mounted cell debris (Jin et al. 2007). FasL and TRAIL are major effectors for the ligands of death receptors. HCV-infected cells will be eliminated if the cells express high levels of MHC class I with HCV antigen.

Our laboratory has reported that a dsRNA analog (polyI:C) has strong ability to activate NK cells in vivo (Akazawa et al. 2007; Matsumoto and Seya 2008). Two main routes for NK cell activation have been reported. First, DCs secrete several cytokines, such as IL-12, IL-18, IL-15 and IFN-α/β in response to dsRNA, and these mediators act on NK cells (Lucas et al. 2007; Matsumoto et al. 2011). Second, DCs express NK-activating ligands on their cell surface and the ligands make a balance shift to activation of NK cells through cell–cell contact (Ebihara et al. 2010). In mouse studies, IL-15 and cell-surface NK-activating ligands are crucial in polyI:C-mediated NK cell activation (Ebihara et al. 2010; Lucas et al. 2007). A main NK-activating ligand induced by polyI:C is IRF-3-inducing NK-activating molecule (INAM) (Ebihara et al. 2010). In a human system with bone marrow-derived DCs (BMDC) and HCV-infected debris (a source of dsRNA), NK cells are activated by BMDC via the TLR3-TICAM-1 pathway in BMDC (Ebihara et al. 2008). Thus, INAM may be a factor that participates in HCV-derived NK activation.

However, how dsRNA and DAMP modify the maturation of DC in an infectious milieu to induce CD4 and CD8 T cells is largely unknown because the functional properties of DAMP generated in HCV-infected hepatocytes have not been well documented (Azuma et al. 2012). Once antigens are presented on MHC class II in DCs upon internalization of infectious debris, CD4 T cells (Longhi et al. 2009), including Th1, Th2, Th9, Th17, and Tregs, are driven in a sophisticated manner. In this context, DAMP and dsRNA could act as the second signal of TLRs triggering DCs to induce cross-presentation, which leads to mounting Ag on MHC class I and subsequently induce the proliferation of CD8 T cells (CTL) (Caskey et al. 2011). Furthermore, the so-called innate lymphocytes may respond to intrinsic stimuli in an Ag-independent fashion. Thus, the function of nucleic acid sensors for DAMP and dsRNA in the presentation of exogenous antigen by DCs is an issue to be tackled (Caskey et al. 2011). Cross-presentation is enhanced by molecules, such as type I IFN and CD40, and by immune cells, including CD4 T cells, NK cells, and NKT cells (Matsumoto and Seya 2008). Yet, the role of type III IFN in T-cell cross-priming and innate lymphocyte activation are

yet unknown. TLR3/TICAM-1 is a main pathway for inducing cross-presentation in response to dsRNA in DCs (Azuma et al. 2012). PolyI:C or virus dsRNA is an example of the TLR3 ligand, and their cross-presentation-inducing activity was first described by Schulz et al. (2005). The effective adjuvancy of polyI:C has been subsequently reported by Steinman group (Caskey et al. 2011; Longhi et al. 2009); however, no report has been definitively determined which DAMPs participate in cross-presentation and possess latent cross-priming (CTL-inducing) ability.

Why HCV circumvents the host immune system of both innate and acquired arms of the immune system remains an ambiguous question. Hepatocytes stand with unique properties with lipid droplet (LD) and the bile secretion system, where hepatocytes secrete bile into canaliculi, which flows into choledochus. HCV and hepatitis B virus induce smoldering inflammation, which is believed to be a nest of carcinogenesis. These viruses have no common properties in their viral-side factors, but the host factors including hepatocytes are common bases for triggering inflammation. Thus, the host factors are undoubtedly critical in inducing infection-driven inflammation and perhaps initiation of tumor progression (Seya et al. 2012). We speculate that persistent HCV infection, followed by inflammation, is caused by the immune aberration involved in HCV infections. The main factors in the innate arm of immunity are DAMP and dsRNA, each of which is reported to associate with smoldering inflammation. However, it is unknown what occurs in the liver if this combined stimulation is constitutively exerted in HCV-infected cells and myeloid cells in the liver. Examining the function of dsRNA and DAMP on chronic HCV infection with increasing studies in innate immunity, inflammation, and cell death, will help us extending our knowledge on vaccine and adjuvants against HCV infection and tumorigenesis. Further molecular analysis will provide a hint for therapeutic strategies for patients who do not respond to IFN therapy.

Acknowledgments We thank Drs. H. Takaki, M. Nakai, R. Takemura, S. Takahashi, L. C. Ring, A. Maruyama, J. Kasamatsu, H. Shime, and M. Azuma in our laboratory for their fruitful discussions. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research, MEXT) and the Ministry of Health, Labor, and Welfare of Japan, and by the Takeda and the Waxmann Foundations. Financial supports by a MEXT Grant-in-Project "the Carcinogenic Spiral", "the National Cancer Center Research and Development Fund (23-A-44)", and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) are gratefully acknowledged.

References

Akazawa T, Ebihara T, Okuno M et al (2007) Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci USA 104:252–257

Andersson A, Yang SC, Huang M et al (2009) IL-7 promotes CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer. J Immunol 182:6951–6958

Angus AGN, Dalrymple D, Boulant S et al (2010) Requirement of cellular DDX3 for hepatitis C virus replication is unrelated to its interaction with the viral core protein. J Gen Virol 91:122–132

Antonelli A, Ferri C, Ferrari SM et al (2008) Immunopathogenesis of HCV-related endocrine manifestations in chronic hepatitis and mixed cryoglobulinemia. Autoimmun Rev 8:18–23

Antonelli A, Ferri C, Ferrari SM et al (2009) Endocrine manifestations of hepatitis C virus infection. Nat Clin Pract Endocrinol Metab 5:26–34

Apolinario A, Majano PL, Lorente R et al (2005) Gene expression profile of T-cell-specific chemokines in human hepatocyte-derived cells: evidence for a synergistic inducer effect of cytokines and hepatitis C virus proteins. J Viral Hepat 12:27–37

Ariumi Y, Kuroki M, Abe K et al (2007) DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J Virol 81:13922–13926

Azuma M, Ebihara T, Oshiumi H et al (2012) Cross-priming for antitumor CTL induced by soluble Ag + polyI:C depends on the TICAM-1 pathway in mouse CD11c+/CD8a+ dendritic cells. Oncoimmunology 1:581–592

Bantel H, Schulze-Osthoff K (2003) Apoptosis in hepatitis C virus infection. Cell Death Differ 10(Suppl 1):S48–S58

Binder M, Kochs G, Bartenschlager R et al (2007) Hepatitis C virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. Hepatology 46:1365–1374

Bortoluci KR, Medzhitov R (2010) Control of infection by pyroptosis and autophagy: role of TLR and NLR. Cell Mol Life Sci 67:1643–1651

Caskey M, Lefebvre F, Filali-Mouhim A et al (2011) Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. J Exp Med 208:2357–2366

Chang PC, Chi CW, Chau GY et al (2006) DDX3, a DEAD box RNA helicase, is deregulated in hepatitis virus-associated hepatocellular carcinoma and is involved in cell growth control. Oncogene 25:1991–2003

Cheng G, Zhong J, Chisari FV (2006) Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. Proc Natl Acad Sci USA 103:8499–8504

Cho YS, Challa S, Moquin D et al (2009) Phosphorylation-driven assembly of the RIP1–RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123

Ding Q, Huang B, Lu J et al (2012) Hepatitis C virus NS3/4A blocks IL-28 production. Eur J Immunol 42:2374–2382

Dixit E, Boulant S, Zhang Y et al (2010) Peroxisomes are signaling platforms for antiviral innate immunity. Cell 141:668–681

Ebihara T, Masuda H, Akazawa T et al (2007) Induction of NKG2D ligands on human dendritic cells by TLR ligand stimulation and RNA virus infection. Int Immunol 19:1145–1155

Ebihara T, Shingai M, Matsumoto M et al (2008) Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. Hepatology 48:48–58

Ebihara T, Azuma M, Oshiumi H et al (2010) Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. J Exp Med 207:2675–2687

Eksioglu EA, Zhu H, Bayouth L et al (2011) Characterization of HCV interactions with Toll-like receptors and RIG-I in liver cells. PLoS One 6:e21186

Estornes Y, Toscano F, Virard F et al (2012) dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. Cell Death Differ 19:1482–1494

Ferreon JC, Ferreon AC, Li K et al (2005) Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. J Biol Chem 280:20483–20492

Florentin J, Aouar B, Dental C et al (2012) HCV glycoprotein E2 is a novel BDCA2 ligand and acts as an inhibitor of IFN production by plasmacytoid dendritic cells. Blood 120:4544–4551

Gack MU, Shin YC, Joo CH et al (2007) TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature 446:916–920

Harada K, Sato Y, Itatsu K et al (2007) Innate immune response to double-stranded RNA in biliary epithelial cells is ssociated with the pathogenesis of biliary atresia. Hepatology 46:1146–1154

He S, Wang L, Miao L et al (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111

He S, Liang Y, Shao F et al (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc Natl Acad Sci USA 108:20054–20059

Hogbom M, Collins R, van den Berg S et al (2007) Crystal structure of conserved domains 1 and 2 of the human DEAD-box helicase DDX3X in complex with the mononucleotide AMP. J Mol Biol 372:150–159

Horner SM, Liu HM, Park HS et al (2011) Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. Proc Natl Acad Sci USA 108:14590–14595

Jelinek I, Leonard JN, Price GE et al (2011) TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. J Immunol 186:2422–2429

Jin B, Wang RY, Qiu Q et al (2007) Induction of potent cellular immune response in mice by hepatitis C virus NS3 protein with double-stranded RNA. Immunology 122:15–27

Kanda T, Steele R, Ray R et al (2009) Inhibition of intrahepatic gamma interferon production by hepatitis C virus nonstructural protein 5A in transgenic mice. J Virol 83:8463–8469

Kawai T, Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 21:317–337

Khvalevsky E, Rivkin L, Rachmilewitz J et al (2007) TLR3 signaling in a hepatoma cell line is skewed towards apoptosis. J Cell Biochem 100:1301–1312

Kim YS, Lee SG, Park SH et al (2001) Gene structure of the human DDX3 and chromosome mapping of its related sequences. Mol Cells 12:209–214

Kono H, Rock KL (2008) How dying cells alert the immune system to danger. Nat Rev Immunol 8:279–289

Larrubia JR, Benito-Martinez S, Calvino M et al (2008) Role of chemokines and their receptors in viral persistence and liver damage during chronic hepatitis C virus infection. World J Gastroenterol 14:7149–7159

Li K, Foy E, Ferreon JC et al (2005a) Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci USA 102:2992–2997

Li XD, Sun L, Seth RB et al (2005b) Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci USA 102:17717–17722

Li K, Li NL, Wei D et al (2012) Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. Hepatology 55: 666–675

Lim EJ, Chin R, Angus PW et al (2012) Enhanced apoptosis in post-liver transplant hepatitis C: effects of virus and immunosuppressants. World J Gastroenterol 18:2172–2179

Lindenbach BD, Thiel HJ, Rice CM (2007) Flaviviridae: the viruses and their replication. In: Knipe DM, Howley PM (eds) Fields virology. Lippincott Williams & Wilkins, Philadelphia, pp 1117–1118

Liu HM, Loo YM, Horner SM et al (2012) The mitochondrial targeting chaperone 14–3-3ε regulates a RIG-I translocon that mediates membrane association and innate antiviral immunity. Cell Host Microbe 11:528–537

Longhi MP, Trumpfheller C, Idoyaga J et al (2009) Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med 206:1589–1602

Loo YM, Owen DM, Li K et al (2006) Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci USA 103:6001–6006

Lucas M, Schachterle W, Oberle K et al (2007) Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity 26:503–517

Matsumoto M, Seya T (2008) TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 60:805–812

Matsumoto M, Oshiumi H, Seya T (2011) Antiviral responses induced by the TLR3 pathway. Rev Med Virol. doi:10.1002/rmv.680

McCartney S, Vermi W, Gilfillan S et al (2009) Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J Exp Med 206: 2967–2976

McLauchlan J, Lemberg MK, Hope G et al (2002) Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J 21:3980–3988

Meylan E, Burns K, Hofmann K et al (2004) RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 5:503–507

Morosky SA, Zhu J, Mukherjee A et al (2011) Retinoic acid-induced gene-I (RIG-I) associates with nucleotide-binding oligomerization domain-2 (NOD2) to negatively regulate inflammatory signaling. J Biol Chem 286:28574–28583

Mulhern O, Bowie AG (2010) Unexpected roles for DEAD-box protein 3 in viral RNA sensing pathways. Eur J Immunol 40:933–935

Nace G, Evankovich J, Eid R et al (2012) Dendritic cells and damage-associated molecular patterns: endogenous danger signals linking innate and adaptive immunity. J Innate Immun 4:6–15

Nakamura M, Funami K, Komori A et al (2008) Increased expression of Toll-like receptor 3 in intrahepatic biliary epithelial cells at sites of ductular reaction in diseased livers. Hepatol Int 2: 222–230

Nitta S, Sakamoto N, Nakagawa M et al (2012) Hepatitis C virus NS4B protein targets STING and abrogates RIG-I-mediated type-I interferon-dependent innate immunity. Hepatology. doi: 10.1002/hep.26017

Oda S, Schroder M, Khan AR (2009) Structural basis for targeting of human RNA helicase DDX3 by poxvirus protein K7. Structure 17:1528–1537

Okamoto K, Moriishi K, Miyamura T et al (2004) Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J Virol 78:6370–6380

Oshiumi H, Sasai M, Shida K et al (2003) TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like

receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 278:49751–49762
Oshiumi H, Matsumoto M, Hatakeyama S et al (2009) Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-beta induction during the early phase of viral infection. J Biol Chem 284:807–817
Oshiumi H, Ikeda M, Matsumoto M et al (2010a) Hepatitis C virus core protein abrogates the DDX3 function that enhances IPS-1-mediated IFN-beta induction. PLoS One 5:e14258
Oshiumi H, Sakai K, Matsumoto M et al (2010b) DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-beta-inducing potential. Eur J Immunol 40:940–948
Oshiumi H, Miyashita M, Inoue N et al (2010c) The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. Cell Host Microbe 8:496–509
Owsianka AM, Patel AH (1999) Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. Virology 257:330–340
Rathinam VA, Fitzgerald KA (2011) Cytosolic surveillance and antiviral immunity. Curr Opin Virol 1:455–462
Saeed M, Shiina M, Date T et al (2011) In vivo adaptation of hepatitis C virus in chimpanzees for efficient virus production and evasion of apoptosis. Hepatology 54:425–433
Saito T, Owen DM, Jiang F et al (2008) Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature 454:523–527
Sawa Y, Arima Y, Ogura H et al (2009) Hepatic interleukin-7 expression regulates T cell responses. Immunity 30:447–457
Schoggins JW, Wilson SJ, Panis M et al (2011) A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 472:481–485
Schroder M (2009) Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation. Biochem Pharmacol 79:297–306
Schroder M, Baran M, Bowie AG et al (2008) Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. EMBO J 27:2147–2157
Schulz O, Diebold SS, Chen M et al (2005) Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 433:887–892
Seth RB, Sun L, Ea CK et al (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell 122:669–682
Seya T, Matsumoto M (2009) The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. Cancer Immunol Immunother 58:1175–1184
Seya T, Shime H, Takaki H et al (2012) TLR3/TICAM-1 signaling in RIP3 tumor necroptosis. Oncoimmunology 1:917–923
Shimoda S, Harada K, Niiro H et al (2008) Biliary epithelial cells and primary biliary cirrhosis: the role of liver-infiltrating mononuclear cells. Hepatology 47:958–965
Shimoda S, Harada K, Niiro H et al (2011) Interaction between Toll-like receptors and natural killer cells in the destruction of bile ducts in primary biliary cirrhosis. Hepatology 53:1270–1281
Sillanpaa M, Kaukinen P, Melen K et al (2008) Hepatitis C virus proteins interfere with the activation of chemokine gene promoters and downregulate chemokine gene expression. J Gen Virol 89:432–443
Soulat D, Burckstummer T, Westermayer S et al (2008) The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response. EMBO J 27:2135–2146
Takaoka A, Taniguchi T (2008) Cytosolic DNA recognition for triggering innate immune responses. Adv Drug Deliv Rev 60:847–857
Takaoka A, Yanai H, Kondo S et al (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 434:243–249
Tanabe M, Kurita-Taniguchi M, Takeuchi K et al (2003) Mechanism of up-regulation of human Toll-like receptor 3 secondary to infection of measles virus-attenuated strains. Biochem Biophys Res Commun 311:39–48
Thomas E, Gonzalez VD, Li Q et al (2012) HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. Gastroenterology 142:978–988
Uematsu S, Akira S (2007) Toll-like receptors and type I interferons. J Biol Chem 282:15319–15323
Wang N, Liang Y, Devaraj S et al (2009) Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. J Virol 83:9824–9934
Wen C, He X, Ma H et al (2008) Hepatitis C virus infection downregulates the ligands of the activating receptor NKG2D. Cell Mol Immunol 5:475–478
Yeretssian G (2012) Effector functions of NLRs in the intestine: innate sensing, cell death, and disease. Immunol Res 54:25–36
Yoneyama M, Kikuchi M, Natsukawa T et al (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5:730–737
Yoneyama M, Onomoto K, Fujita T (2008) Cytoplasmic recognition of RNA. Adv Drug Deliv Rev 60:841–846
Zeremski M, Petrovic LM, Talal AH (2007) The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection. J Viral Hepat 14:675–687
Zhang Z, Kim T, Bao M et al (2011) DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. Immunity 34:866–878
Zhu H, Dong H, Eksioglu E et al (2007) Hepatitis C virus triggers apoptosis of a newly developed hepatoma cell line through antiviral defense system. Gastroenterology 133:1649–1659

ELSEVIER

Institut Pasteur

Microbes and Infection xx (2013) 1–10

www.elsevier.com/locate/micinf

Original article

Systemic biological analysis of the mutations in two distinct HIV-1mt genomes occurred during replication in macaque cells

Masako Nomaguchi [a], Naoya Doi [a], Sachi Fujiwara [a], Akatsuki Saito [b], Hirofumi Akari [b], Emi E. Nakayama [c], Tatsuo Shioda [c], Masaru Yokoyama [d], Hironori Sato [d], Akio Adachi [a,*]

[a] *Department of Microbiology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan*
[b] *Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Aichi, Japan*
[c] *Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*
[d] *Laboratory of Viral Genomics, Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan*

Received 17 December 2012; accepted 24 January 2013

Abstract

Fundamental property of viruses is to rapidly adapt themselves under changing conditions of virus replication. Using HIV-1 derivatives that poorly replicate in macaque cells as model viruses, we studied here mechanisms for promoting viral replication in non-natural host cells. We found that the HIV-1s could evolve to grow better in both macaque and human cells by the continuous culture in macaque lymphocyte cell lines. Notably, only several mutations at defined sites of the Pol-integrase and/or the Env-gp120 reproducibly appeared in repeated adaptation experiments and were sufficient to cause the phenotypic change. Meanwhile, no amino acid changes to enhance viral replication in macaque cells were found in interaction sites for the known anti-retroviral proteins. These findings disclose a hitherto unappreciated evolutionary pathway to augment HIV-1 replication in primate cells, where tuning of viral interactions with positive rather than negative factors for replication can play a dominant role.

Keywords: HIV-1; HIV-1mt; Pol-IN; Env-gp120; Adaptive mutation; Macaque cells

1. Introduction

Viruses evolve extremely rapidly under the changing conditions of virus replication. HIV-1 is no exception. HIV-1 possesses high adaptation potential due to the ability to acquire sequence alterations through high mutation rate of reverse transcriptase (RT) and recombination of viral genomes [1,2]. Change of viral properties by genetic alterations leads to resistance to antiviral drugs, escape from host immune system, and adaptation to new hosts upon transmission [3–6]. Experimental approaches that analyze the genomic change and evolution of viruses are commonly used effective measures to know how viruses adapt themselves under a certain selective pressure. Such studies, however, usually have focused on genetic changes in a limited and selected region of viral genomes or sequence variations in a specified mass of virus.

HIV-1 does not replicate in most animal species including rodents and macaques. Inhibition of HIV-1 replication in macaque cells, at least in part, is mediated by host restriction factors such as APOBEC3 proteins, cyclophilin A (CypA), TRIM5α/TRIMCyp (TRIM5 proteins), and tetherin (for review, refer to references [7–10]). Encounters with pathogenic viruses impose selective pressure on restriction factors, and influence their antiviral specificity [11–13]. Even though human cells also have orthologs of these factors, HIV-1 evades their restriction and replicates well in humans. This suggests that both viruses and host cells co-evolve under the mutual selective pressure. Thus, evolution of viruses is determined by adaptation potential of viruses and their interaction with host cells.

* Corresponding author. Tel.: +81 88 633 7078; fax: +81 88 633 7080.
E-mail address: adachi@basic.med.tokushima-u.ac.jp (A. Adachi).

1286-4579/$ - see front matter © 2013 Published by Elsevier Masson SAS on behalf of Institut Pasteur.
http://dx.doi.org/10.1016/j.micinf.2013.01.005

Prototype macaque cell-tropic HIV-1 (pHIV-1mt), NL-DT5R (5R; X4-tropic) and NL-DT562 (562; R5-tropic), carry a small portion of *gag* gene and an entire *vif* gene from SIVmac239 (Fig. 1). These viruses are insufficiently infectious for macaque cells by their partial resistance to CypA/TRIM5 proteins and evasion from APOBEC3G/F restriction, and their replication potentials were significantly lower than that of pathogenic SIVmac239 [14–16]. These results indicate that macaque cells still impose strong restrictive pressure on pHIV-1mt replication. Therefore, clones 5R and 562 are suitable materials to analyze what genetic changes they would acquire and how they would adapt themselves to environments within macaque cells. As experimental approach to do this, we have performed virus adaptation experiments by the long-term culture of 5R- or 562-infected macaque cells, and successfully obtained adapted viruses with enhanced growth ability [17]. In this study, we inquired into the biological relevance of mutations that appeared in the genomes of adapted viral clones by investigating thoroughly the effect of mutations on viral growth. Although a number of genetic substitutions were found in the genomes of adapted viruses, mutations in *pol*-integrase (IN) and *env*-gp120 only were found to be responsible for enhancement of viral replication. Extensive and repeated virus adaptation experiments revealed that viral clones with augmented growth potential are characterized by acquisition of adaptive mutations in *pol*-IN and in *env*-gp120. Our results suggest that pHIV-1mt evolves in a certain adaptation pathway under the restrictive and uniform environments imposed by macaque cells, and that genetic alterations do not always have biological significance for escaping selective pressure, supporting Kimura's neutral theory of molecular evolution [18].

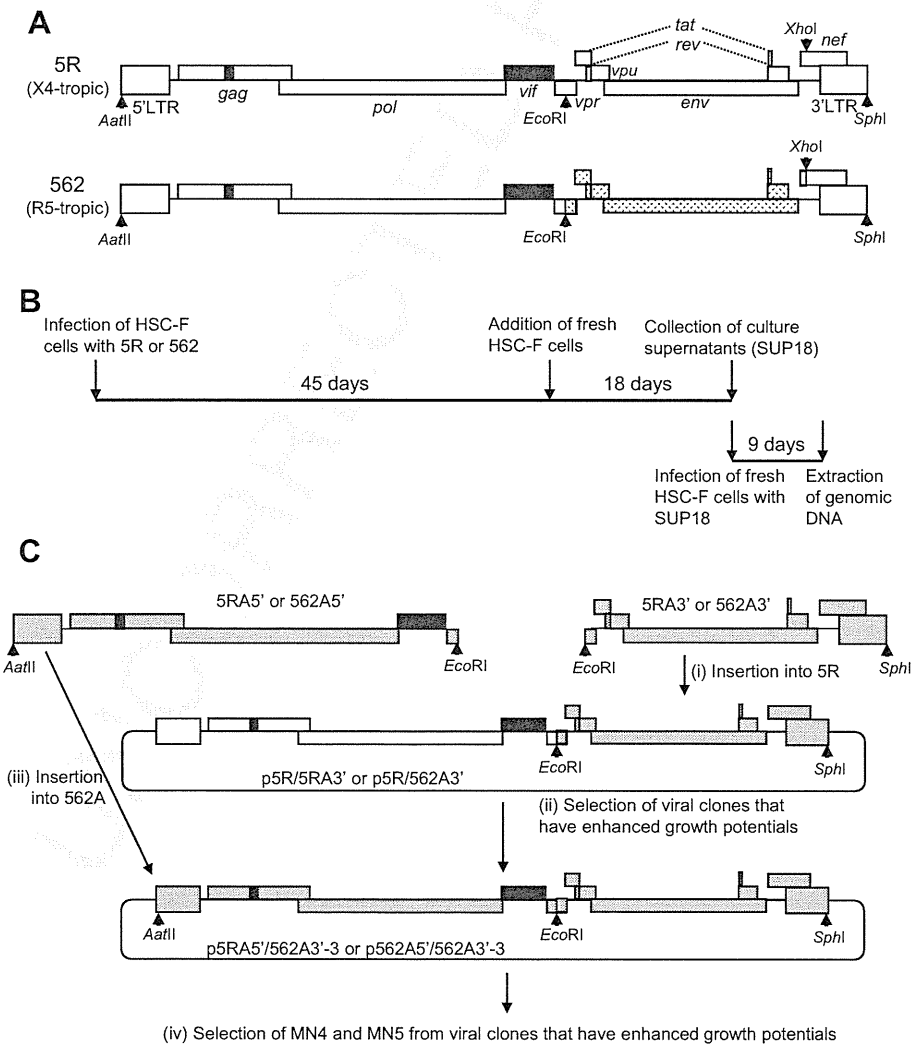

Fig. 1. Molecular cloning of viral genomes from macaque cell-adapted viruses. (A) Genome structure of 5R and 562 [14,16]. Sequence from NF462 [32] is shown by dotted areas. Black areas show the regions derived from SIVmac239. (B) Schedule of virus adaptation and the harvest of adapted viruses [17]. Genomic DNA prepared from infected cells for molecular cloning is indicated. (C) Generation and selection of proviral clones from adapted 5R and 562 viruses. The procedure starts with (i) and ends with (iv). For details, see Materials and methods. PCR amplified fragments derived from adapted 5R (5RA) or adapted 562 (562A) are shown by gray areas. White and black areas indicate the region from 5R.

Please cite this article in press as: M. Nomaguchi, et al., Systemic biological analysis of the mutations in two distinct HIV-1mt genomes occurred during replication in macaque cells, Microbes and Infection (2013), http://dx.doi.org/10.1016/j.micinf.2013.01.005