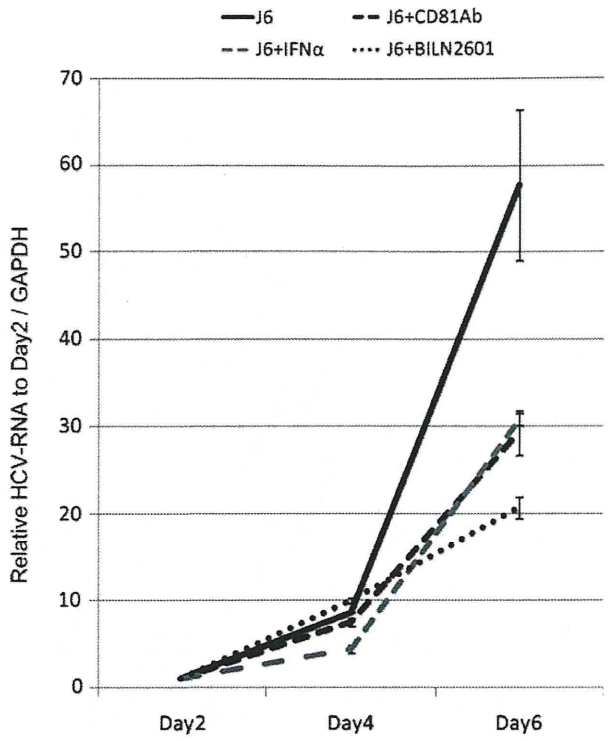

FIG. 2. J6JFH1 B-cell infection is blocked by anti-CD81 Ab, IFN-α, or an NS3/4A inhibitor. Anti-CD81 neutralizing Ab (20 μg/mL) was added to the B-cell culture 1 h before infection. Otherwise, recombinant IFN-α rhIFN-α, 200 IU/mL) or BLIN2601 (250 nM, which is IC75; see Supplementary Fig. S3) was added 1 h after infection. On days 2, 4, and 6, total RNA was extracted, and HCV-RNA was determined by RT-PCR. The values were adjusted by GAPDH.

may reflect the fact that the NS5A protein is difficult to detect in infected B-cells using IF assay.

B-cells can be infected with different HCV strains

We next used the Jc1/GLuc2A strain to investigate whether different HCV strains infect primary B-cells. Primary B-cells, non-B-cells (data not shown), and Huh7.5.1 cells were infected with the Jc1/GLuc2A strain. After five washes, supernatant was collected (day 0 samples). On days 2, 4, and 6, medium was collected. Luciferase activity was determined for all samples by luminescence (GLuc). GLuc activity and detection of RNA increased exponentially in Huh7.5.1 cells infected with the Jc1/GLuc2A strain (Fig. 4A). GLuc activity on day 4 to day 6 increased more in primary B-cells than in non-B-cells (Fig. 4B). These results suggest that HCV replication is substantial, but low in the HCV line Jc1/GLuc2A.

B-cells neither produce nor release detectable level of HCV infectious particles

We collected supernatants of J6JFH1-infected primary human B-cells to measure productive infection in B-cells. The supernatant was then added to culture of Huh7.5.1 cells, and we compared infection with control Huh7.5.1 cells, whose cells were infected with a low MOI (0.01 and 0.001) of J6JFH1 collected from media of the infected Huh7.5.1 cells. HCV-RNA titer in the Huh7.5.1 titrating cells was decreased over time after co-culture with B-cell supernatants obtained from either "releasing samples" "assembly samples." In contrast, HCV-RNA titers were slightly increased over time in the Huh7.5.1 titrating cells that had been infected with medium collected from low MOI-J6JFH1-infected Huh7.5.1 cells (Fig. 5). These results indicated that primary human B-cells were infected with J6JFH1 but failed to assemble or produce particles into the supernatant.

Host response to HCV infection into primary B-cells

Next, we determined whether B-cell activation was induced in HCV-infected B-cells that survived under HCV infection. We measured induction of CD80 and CD86 as B-cell activation markers. After 2–3 days of infection, the CD80/86 levels on B-cells treated with J6JFH1 were compared with those treated with medium from mock-infected cells (concentrated Huh7.5.1 medium) by FACS analysis (Fig. 6A). We found that CD80/86 were upregulated in infected cells compared to mock-infected cells.

Since B-cell lymphoma is a known complication of chronic HCV infection (20,36) and acquiring apoptotic resistance is essential for the development of cancer (21,51,38), we measured the ability of B-cells to escape apoptosis after HCV infection. B-cell apoptosis spontaneously occurs during culture at 37°C. The percent of apoptosis of primary B-cells was decreased in FACS analysis using 7AAD viaprobe + annexinV (Fig. 6B) and ATP assays postinfection (Fig. 6C). These results suggest that primary B-cells are protected from apoptosis by infection with HCVcc. It has been reported that B-cells were vulnerable to apoptotic cell death at various stages of peripheral differentiation and during signal responses (18). Thus, the results infer that HCV stimulation interferes with B-cell apoptotic signal in human B-cells.

Discussion

We show evidence suggesting that human peripheral B-cells can be infected with HCV strains. Establishment of J6JFH1 infection was evaluated by minus-strand PCR amplification, production of core and NS5A proteins, and protection from apoptosis. An increase in HCV RNA in B-cells was inhibited by an exogenously added antibody against CD81 that blocked HCV receptor function. Furthermore, blocking HCV replication in B-cells by type I IFN and NS3/4A protease inhibitor confirmed the presence of HCV infection/replication in human B-cells. The results were corroborated with another HCV strain, Jc1/GLuc2A. Although we failed to establish an EBV-transformed B-cell line to reproduce HCV infection of B-cells, peripheral blood B-cells were infected with J6JFH1 in 12 independent experiments.

One of the well-known complications of chronic HCV infection is LPD, including cryoglobulinemia and B-cell malignant lymphoma, indicating the involvement of B-cells in the course of the disease (1,12,15,16). However, many reports describing the existence of the HCV genome in B-cells and lymphomas (21,25,51) and HCV replication in B-cells have been controversial due to multiple artifacts complicated in detection and quantitation of the replicative

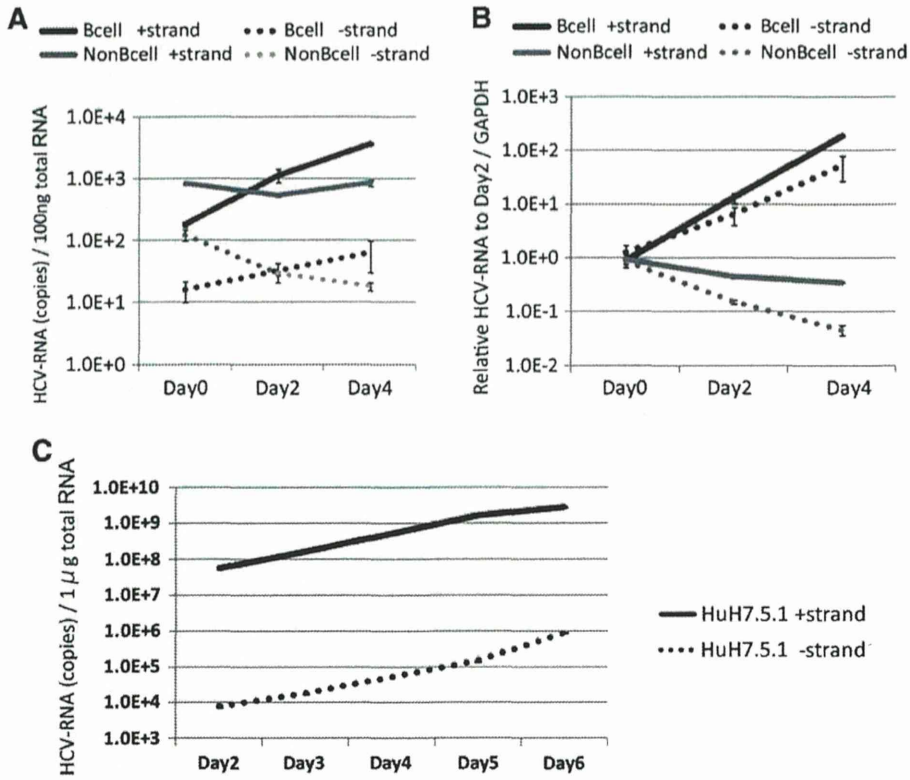

FIG. 3. HCV negative strand RNA is detected in human B-cells. By using rTth methods, HCV strand-specific RNA was determined in J6JFH1-infected human B-cells. **(A)** Not only plus strand HCV-RNA but also minus strand HCV-RNA were increased in a time-dependent manner in human B-cells. **(B)** When HCV-RNA was adjusted by GAPDH that was used as an internal control, HCV-RNAs in B-cells were substantially increased compared with those in non-B-cells. **(C)** Plus and minus strand HCV-RNAs were efficiently amplified in J6JFH1-infected Huh 7.5.1 cells. The level of HCV-RNA exponentially increased in this hepatocyte line.

intermediate minus strand RNA (29,31). This has led to a continuous debate about HCV infection in B-lymphocytes.

HCV entry into B-cells has also been previously reported to be absent because retroviral (37) and lentiviral (8) pseudoparticles bearing HCV envelope glycoproteins (HCVpp) did not infect primary B-cells or B-cell lines. In our study, while we succeeded in infecting Huh7.5.1 cells efficiently with retroviral pseudoparticles for expressing both HCV E1/E2 and the control VSV-G, we failed to establish the same infection in B-cells, suggesting that the block of pseudoparticle entry into B-cells is not related to HCV glycoproteins alone.

Total PBMCs reportedly facilitate HCV attachment but not internalization (42), so HCV infection of B-cells is abrogated in total PBMCs (35). The cause of HCV absorption is unclear, but incomplete sets of HCV receptors in non-B PBMC cells permit attachment of HCV without internalization. B-cells possess CD81, SRBI, LDL-R, and NPC1L1. Because B-cells are not adherent cells, they do not express claudin 1 and occludin, which forms a receptor complex for HCV (9,14,19,43). Claudin 1 and occludin are components of tight junctions and serve as HCV receptors in human hepatocytes. In infection studies using cells expressing these proteins, however, claudin 1 and occludin only upgrade infection efficacy and are dispensable to infection (5), although CD81 is essential for establishment of infection (42). Lack of claudin 1 and occludin or miR122 might be a cause of the low HCV infection efficiency observed in human B-cells. Function blocking of CD81 by its specific antibody suppressed HCV infection in primary B-lymphocytes, which imply that HCV entry into primary B-lymphocyte is dependent on the direct interaction phenomenon between HCV virus particles and CD81 receptor and is not mediated by other nonspecific (CD81 independent) pathways such as exosomal transfer of HCV from Huh7 cells to nonhepatic cells, such as dendritic cells (46).

Previous report using *in vitro* prepared recombinant HCV JFH1 particles (HCVcc) failed to establish HCV infection in B-lymphocyte cell lines (39). While HCV is known to infect human hepatocytes *in vivo* leading to chronic viral hepatitis, in the *in vitro* conditions, only the combination between Huh7 cells and its derived clones supported robust replication and infection with only JFH1 or its derived chimeras (5). Neither hepatocyte cell lines including primary hepatocytes nor other HCV strains could reproduce HCV infection efficiently *in vitro* (5). These data suggest that the clonal selection of HCV quasispecies by hepatoma Huh7 cells is essential for this robust infection *in vitro*. The situation would be similar to the JFH1 story in B-cell HCV infection.

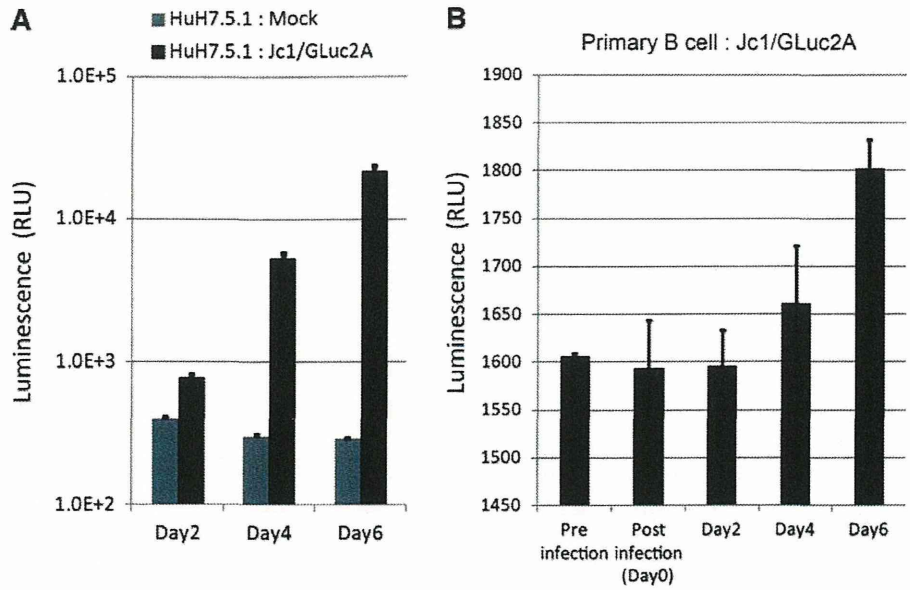

FIG. 4. Jc1/GLuc2A strain infects human B-cells with an increase of Gluc activity. Human B-cells and Huh7.5.1 cells were infected with the JC1/GLuc2A strain that contains secretory luciferase derived from *Gaussia* (GLuc) at MOI = 5. Huh7.5.1 cells were used as control. GLuc activity was increased as time cultured. The GLuc activity was saturated in Huh7.5.1 (**A**). On the other hand, GLuc activity was increased from day 4 in human B-cells (**B**).

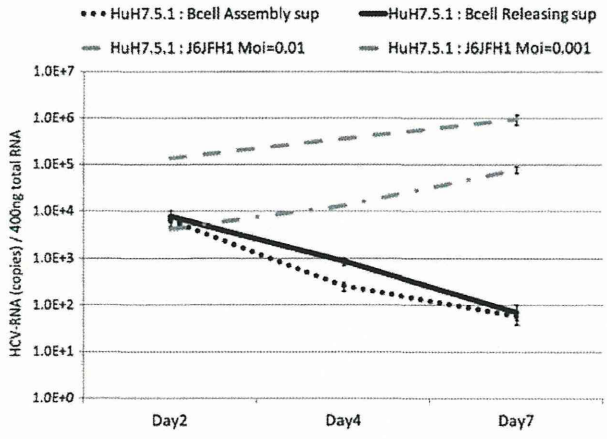

FIG. 5. B-cells infected with J6JFH1 fail to produce virus particles. Human B-cells were infected with J6JFH1 for 3 h, washed twice with phosphate buffered saline (PBS), and cultured. Six days after infection, the supernatant was collected ("releasing samples"). Cells were periodically frozen and thawed five times, and the supernatant was collected ("assembly samples"). For evaluation of the infectious virions, Huh7.5.1 cells were treated with these "releasing samples" or "assembly samples." Similarly, Huh7.5.1 cells were treated with J6JFH1 at low MOI (MOI = 0.01 and 0.001) in parallel. After the treatment, cells were washed and cultured. On days 2, 4, and 7, cells were harvested to collect HCV-RNA. Total RNA was extracted from each samples, and HCV-RNA was determined by RT-PCR methods.

B-cell apoptosis spontaneously occurs during culture at 37°C. We found that B-cell apoptosis was blocked by J6JFH1 infection, as reported previously using Raji cells (11). B-cell apoptosis usually occurs secondary to viral infection, but HCV is particular since apoptotic signaling interferes with infection, leading to protection from cell death. However, B-cell survival was not due to primary infection, because the percent of cells circumventing apoptosis was usually higher than cells infected with HCV. We could not define the pathways that participated in apoptosis regulation by HCV, although a previous report (11) suggested that E2-CD81 engagement was related to B-lymphocyte disorders and weak neutralizing antibody response in HCV patients. Since B-cell lymphoma is a known complication of chronic HCV infection (27), the inability of infected cells to undergo apoptosis can be associated with the development of cancer (28,33,49). In this context, B-cell lymphoma often occurs in mice with Cre-initiated HCV transgenes (26). It is notable that anti-apoptotic effect of HCV core gene was reported in genotype 3a in Huh7 cells (23) and, here, genotype 2a in B-cells. In another report (51), HCV strains established from B-cell lymphoma persistently infected with HCV were genotype 2b. B-cell HCV infection might not be linked to some specific genotypes of HCV.

We believe that our report shows that human primary B-cells can be infected *in vitro* with HCV, and that this infection is dependent on HCV particles binding with its receptor CD81 and is not nonspecific entry (e.g., exosomal mediated). We also show that this infection could be blocked with antibodies interfering with this binding, or with drugs that suppress HCV replication. Although no virion was generated from B-cells in HCV infection, it is still likely that B-cells serve as a temporal reservoir of HCV in the blood circulation. If B-cells permit HCV infection, RNA sensors

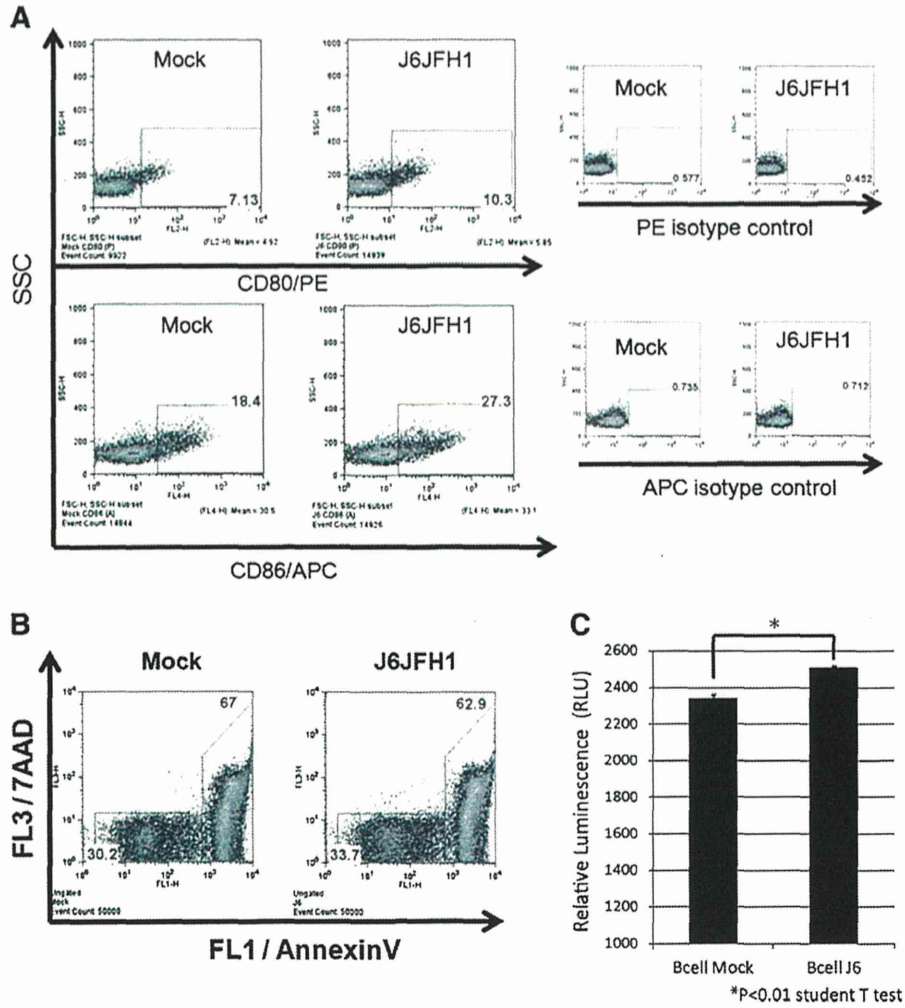

FIG. 6. J6JFH1 infection activates B-cells and protects the cells from apoptosis. Human B-cells were infected with J6JFH1 at MOI = 1 for 3 h, washed twice with PBS, and cultured. Two days after inoculation, cells were washed and suspended with FACS buffer. **(A)** The cells were incubated with PE-conjugated anti-human CD80 antibody, APC-conjugated CD86 antibody, or PE/APC-conjugated mouse IgG1 isotype control for 30 min. Then, the cells were washed and resuspended in FACS buffer. Cells were analyzed by FACS. **(B)** Annexin V and 7AAD viaprobe were added and cultured at 18°C for 10 min. Then, cells were analyzed by FACS. **(C)** 2×10^5 human B-cells were infected with J6JFH1- or Mock-concentrated medium for 3 h. Cells were then washed, resuspended, and cultured in a 96-well white microwell plate. Two days later, ATP activity was determined with a CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega). ATP activity was adjusted by day 0 ATP activity.

RIG-I and MDA5 in B-cells might recognize HCV RNA and evoke intracellular signaling, including by transcription factors NF-κB and IRF-3/7 (5). Activation of the cytokine network is triggered in human B-cells in response to HCV RNA. In fact, host factors liberated by HCV-infecting B-cells have been previously reported in HCV patients (1,12,15,16,52). Although patients' outcomes would be more than we can be predicted from our results, this system would actually benefit the future study on B-cell–virus interaction.

Acknowledgments

We are grateful to Drs. Frank Chisari (Scripps Research Institute, San Diego, CA) for the Huh7.5.1 cells, Takaji Wakita (National Institute of Infectious Diseases, Tokyo) for supplying the J6JFH1 plasmid, and Brett Lindenbach (Yale University, New Haven, CT) for providing us with the pJc1-GLuc2A HCV strain.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for "Carcinogenic Spiral") and the Ministry of Health, Labor, and Welfare of Japan, and by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT. Financial support by the Takeda Science Foundation, the Yasuda Cancer Research Foundation, and the Iskra Foundation are gratefully acknowledged.

Author Disclosure Statement

No competing financial interests exist.

References

1. Agnello V, Chung RT, and Kaplan LM. A role for hepatitis C virus infection in type II cryoglobulinemia. N Engl J Med 1992;327:1490–1495.
2. Aizaki H, Morikawa K, Fukasawa M, *et al.* Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 2008;82:5715–5724.
3. Aly HH, Oshiumi H, Shime H, *et al.* Development of mouse hepatocyte lines permissive for hepatitis C virus (HCV). PLoS One 2011;6:e21284.
4. Aly HH, Qi Y, Atsuzawa K, *et al.* Strain-dependent viral dynamics and virus–cell interactions in a novel *in vitro* system supporting the life cycle of blood-borne hepatitis C virus. Hepatology 2009;50:689–696.
5. Aly HH, Shimotohno K, Hijikata M, and Seya T. *In vitro* models for analysis of the hepatitis C virus life cycle. Microbiol Immunol 2012;56:1–9.
6. Asselah T, and Marcellin P. Second-wave IFN-based triple therapy for HCV genotype 1 infection: simeprevir, faldaprevir and sofosbuvir. Liver Int 2014;34:60–68.
7. Bare P, Massud I, Parodi C, *et al.* Continuous release of hepatitis C virus (HCV) by peripheral blood mononuclear cells and B-lymphoblastoid cell-line cultures derived from HCV-infected patients. J Gen Virol 2005;86:1717–1727.
8. Bartosch B, Dubuisson J, and Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 2003;197; 633–642.
9. Bartosch B, Vitelli A, Granier C, *et al.* Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 2003;278:41624–41630.
10. Castet V, Fournier C, Soulier A, *et al.* Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected *in vitro*. J Virol 2002;76:8189–8199.
11. Chen Z, Zhu Y, Ren Y, *et al.* Hepatitis C virus protects human B lymphocytes from Fas-mediated apoptosis via E2-CD81 engagement. PLoS One 2011;6:e18933.
12. Donada C, Crucitti A, Donadon V, *et al.* Systemic manifestations and liver disease in patients with chronic hepatitis C and type II or III mixed cryoglobulinaemia. J Viral Hepat 1998;5:179–185.
13. Ebihara T, Shingai M, Matsumoto M, Wakita T, and Seya T. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. Hepatology 2008;48:48–58.
14. Evans MJ, von Hahn T, Tscherne DM, *et al.* Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007;446:801–805.
15. Ferri C, Caracciolo F, Zignego AL, *et al.* Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. Br J Haematol 1994;88:392–394.
16. Frangeul L, Musset L, Cresta P, Cacoub P, Huraux JM, and Lunel F. Hepatitis C virus genotypes and subtypes in patients with hepatitis C, with and without cryoglobulinemia. J Hepatol 1996;25:427–432.
17. Fried MW, Buti M, Dore GJ, *et al.* Once-daily simeprevir (TMC435) with Pegylated interferon and ribavirin in treatment-naïve genotype 1 hepatitis C: the randomized PILLAR Study. Hepatology 2013;58:1918–1929.
18. Harwood NE, and Batista FD. New insights into the early molecular events underlying B cell activation. Immunity 2008;28:609–619.
19. Hsu M, Zhang J, Flint M, *et al.* Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 2003;100: 7271–7276.
20. Inokuchi M, Ito T, Uchikoshi M, *et al.* Infection of B cells with hepatitis C virus for the development of lymphoproliferative disorders in patients with chronic hepatitis C. J Med Virol 2009;627:619–627.
21. Ito M, Masumi A, Mochida K, *et al.* Peripheral B cells may serve as a reservoir for persistent hepatitis C virus infection. J Innate Immun 2010;2:607–617.
22. Jacobson IM, McHutchison JG, Dusheiko G, *et al.* Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med 2011;364:2405–2416.
23. Jahan S, Khaliq S, Siddiqi MH, *et al.* Anti-apoptotic effect of HCV core gene of genotype 3a in Huh-7 cell line. Virol J 2011;8:522.
24. Kambara H, Fukuhara T, Shiokawa M, *et al.* Establishment of a novel permissive cell line for the propagation of hepatitis C virus by expression of microRNA miR122. J Virol 2012;86:1382–1393.
25. Karavattathayyil SJ, Kalkeri G, Liu HJ, *et al.* Detection of hepatitis C virus RNA sequences in B-cell non-Hodgkin lymphoma. Am J Clin Pathol 2000;113:391–398.
26. Kasama Y, Sekiguchi S, Saito M, *et al.* Persistent expression of the full genome of hepatitis C virus in B cells induces spontaneous development of B-cell lymphomas *in vivo*. Blood 2010;116;4926–4933.
27. Kondo Y, and Shimosegawa T. Direct effects of hepatitis C virus on the lymphoid cells. World J Gastroenterol 2013; 19:7889–7895.
28. Ladu S, Calvisi DF, Conner EA, Farina M, Factor VM, and Thorgeirsson SS. E2F1 inhibits c-Myc-driven apoptosis via PIK3CA/Akt/mTOR and COX-2 in a mouse model of human liver cancer. Gastroenterology 2008;135:1322–1332.
29. Lanford RE, Chavez D, Chisari FV, and Sureau C. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. J Virol 1995;69:8079–8083.
30. Laskus T, Radkowski M, Wang LF, Vargas H, and Rakela J. The presence of active hepatitis C virus replication in lymphoid tissue in patients coinfected with human immunodeficiency virus type 1. J Infect Dis 1998;178:1189–1192.
31. Lerat H, Berby F, Trabaud MA, *et al.* Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J Clin Invest 1996;97:845–851.
32. Lok AS, Gardiner DF, Hézode C, *et al.* Randomized trial of daclatasvir and asunaprevir with or without PegIFN/RBV for hepatitis C virus genotype 1 null responders. J Hepatrol 2014;60:490–499.
33. Lowe SW, and Lin AW. Apoptosis in cancer. Carcinogenesis 2000;21:485–495.
34. MacParland SA, Pham TN, Guy CS, and Michalak TI. Hepatitis C virus persisting after clinically apparent sustained virological response to antiviral therapy retains infectivity *in vitro*. Hepatology 2009;49:1431–1441.
35. Marukian S, Jones CT, Andrus L, *et al.* Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatology 2008;48:1843–1850.
36. Mazzaro C, Franzin F, Tulissi P, *et al.* Regression of monoclonal B-cell expansion in patients affected by mixed cryoglobulinemia responsive to alpha-interferon therapy. Cancer 1996;77:2604–2613.

37. McKeating JA, Zhang LQ, Logvinoff C, *et al.* Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner. J Virol 2004;78:8496–8505.
38. Mizuochi T, Ito M, Takai K, and Yamaguchi K. Peripheral blood memory B cells are resistant to apoptosis in chronic hepatitis C patients. Virus Res 2011;155:349–351.
39. Murakami K, Kimura T, Shoji I, *et al.* Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines. J Gen Virol 2008;89:1587–1592.
40. Muratori L, Gibellini D, Lenzi M, *et al.* Quantification of hepatitis C virus-infected peripheral blood mononuclear cells by *in situ* reverse transcriptase-polymerase chain reaction. Blood 1996;88:2768–2774.
41. Phan T, Beran RKF, Peters C, Lorenz IC, and Lindenbach BD. Hepatitis C virus NS2 protein contributes to virus particle assembly via opposing epistatic interactions with the E1-E2 glycoprotein and NS3-NS4A enzyme complexes. J Virol 2009;83:8379–8395.
42. Pileri P, Uematsu Y, Campagnoli S, *et al.* Binding of hepatitis C virus to CD81. Science 1998;282:938–941.
43. Ploss A, Evans MJ, Gaysinskaya VA, *et al.* Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 2009;457:882–886.
44. Qu J, Zhang Q, Li Y, *et al.* The Tat protein of human immunodeficiency virus-1 enhances hepatitis C virus replication through interferon gamma-inducible protein-10. BMC Immunol 2012;13:15.
45. Radkowski M, Gallegos-Orozco JF, Jablonska J, *et al.* Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. Hepatology 2005;41:106–114.
46. Ramakrishnaiah V, Thumann C, Fofana I, *et al.* Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. Proc Natl Acad Sci U S A 2013;110:13109–13113.
47. Sarhan MA, Pham TNQ, Chen AY, and Michalak TI. Hepatitis C virus infection of human T lymphocytes is mediated by CD5. J Virol 2012:86:3723–3735.
48. Schmidt WN, Stapleton JT, LaBrecque DR, *et al.* Hepatitis C virus (HCV) infection and cryoglobulinemia: analysis of whole blood and plasma HCV RNA concentrations and correlation with liver histology. Hepatology 2000;31:737–744.
49. Schulze-Bergkamen H, Krammer P.H. Apoptosis in cancer—implications for therapy. Semin Oncol 2004;31:90–119.
50. Seto WK, Lai CL, Fung J, *et al.* Natural history of chronic hepatitis C: genotype 1 versus genotype 6. J Hepatol 2010;53:444–448.
51. Sung VM, Shimodaira S, Doughty AL, *et al.* Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus *in vivo* and *in vitro*: the apoptotic effects of virus infection. J Virol 2003;77:2134–2146.
52. Turner NC. Hepatitis C and B-cell lymphoma. Ann Oncol 2003;14:1341–1345.
53. Wakita T, Pietschmann T, Kato T, *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nature Med 2005;11:791–796.

Address correspondence to:
Dr. Tsukasa Seya
Department of Microbiology and Immunology
Hokkaido University Graduate School of Medicine
Kita 15, Nishi 7
Kita-ku
Sapporo 060-8638
Japan

E-mail: seya-tu@pop.med.hokudai.ac.jp

Cell Host & Microbe

Measles Virus Takes a Two-Pronged Attack on PP1

Tsukasa Seya[1,*]
[1]Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University Kita-ku, Kita-15, Nishi-7, Sapporo 060-8638, Japan
*Correspondence: seya-tu@pop.med.hokudai.ac.jp
http://dx.doi.org/10.1016/j.chom.2014.06.013

During viral infection, RIG-I-like receptors (RLRs) are activated upon dephosphorylation by the phosphatase PP1, resulting in type I interferon production. In this issue, Davis et al. (2014) and Mesman et al. (2014) show that measles virus inhibits this antiviral response by targeting PP1 and thus preventing RLR dephosphorylation and activation.

Foreign RNAs are recognized by the innate Toll-like receptor 3 (TLR3) in phago-endosomes and by RIG-I-like receptors (RLR), RIG-I and MDA5, in the cytoplasm (Figure 1). RLRs in infected cells sense viral RNA and its replicative products and trigger the activation of the adaptor MAVS and subsequently the transcription factor IRF-3. These events lead to the production of type I interferon (IFN), which in turn induces antiviral genes and abrogates viral replication. A large body of growing research provides a detailed understanding of the unique features of IRF-3- and IFN-inducible genes in blocking virus replication through various innate immune mechanisms. Despite these sophisticated antiviral responses, viruses have evolved strategies to circumvent the host antiviral IFN system.

As one example, the paramyxovirus measles virus (MV) encodes the V and C genes, which abrogates host innate defense responses. The products of the V and C genes are generated through alternative reading frames or RNA editing of the P protein-encoded region. The P protein is critical for MV replication, while the V and C proteins are accessory proteins dispensable for assembling MV particles, but have an important role in suppressing the host IFN-inducing pathway. In particular, the V protein has been shown to antagonize IFN induction. The mechanism whereby the V protein MV supports viral propagation has been proposed in earlier reports by Childs et al. (2009) and Ikegame et al. (2010). A major target of the MV-V protein is the cytoplasmic DEAD/box helicase, MDA5. The V protein of MV physically interacts with MDA5, but the mechanism by which the V protein interferes with MDA5 activity to prevent type I IFN induction remains unclear. Recent crystal-structure analysis suggested that MV-V protein unfolds MDA5 and binds a structural motif within MDA5 that is normally buried in the helicase fold. This leads to disruption of the MDA5 ATP-hydrolysis site and hinders MDA5 activation.

In this issue, a pair of papers examine how MV disrupts MDA5 and RIG-I activity and show that activation of these RLRs can be prevented by two different mechanisms. Gack and colleagues (Davis et al., 2014) use a series of biochemical studies to show that the V protein inhibits MDA5 by preventing the removal of an inhibitory phosphorylation mark. In a previous report, the authors demonstrated that MDA5 signaling is suppressed by constitutive phosphorylation (Wies et al., 2013). Dephosphorylation by the phosphatase PP1 is an essential step leading to the unfolding and activation of MDA5. In the current study, Gack and colleagues present evidence that V protein forms a complex with PP1α/γ and serves as a substrate for dephosphorylation by PP1, which competitively prevents PP1-mediated dephosphorylation of MDA5 (Figure 1).

This scenario requires that MDA5 be constitutively suppressed by phosphorylation via the action of an unknown kinase; however, how this initial modification of MDA5 occurs has not been investigated. Additionally, as the main conclusions regarding V protein-mediated inhibition of MDA5 were largely drawn using epithelial cell lines (Davis et al., 2014), despite the fact that dendritic cells (DCs) are the initial target during MV infection, it will be important to determine whether MV-V dephosphorylation by PP1 regulates MDA5 activation and type I IFN production in MV-infected patients.

In a related study, Geijtenbeek and colleagues (Mesman et al., 2014) show that MV infection can also target PP1 in DCs via a different mechanism. They find that MV binding to the cell surface lectin DC-SIGN signals the activation of the kinase Raf-1, which leads to the inhibition of phosphatase PP1 (Figure 1). Raf-1 activation is known to facilitate association of the phosphatase inhibitor I-1 with GADD34-PP1 phosphatase holoenzymes, thereby inhibiting its phosphatase activity (Figure 1). This in turn, antagonizes the IFN-induced antiviral response, as the activation of both RIG-I and MDA5 by PP1-dependent dephosphorylation is inhibited. Literature has revealed that DC-SIGN may not act as an entry receptor, but rather plays a role in the attachment of MV (de Witte et al., 2006). The current study further endowed a function of DC-SIGN in controlling the PP1 phosphatase function through Raf-1 signaling.

These reports present pathways through which MV modulates antiviral response of infected cells to promote virus propagation. Myeloid-derived cells like DCs are among the first cells that become infected via the viral entry receptors CD150 (for wild-type MV strains) and, to a lesser extent, CD46 (vaccine strains). These myeloid lineages encompass different cell subsets that express lineage-specific surface molecules. According to a recent report by Takaki et al. (2014), DC subsets exhibit different susceptibility to MV infection in a mouse model. Such distinct features may be the result of the activation of different antiviral pathways, namely the TLR/MyD88 pathway in $CD4^+$ T cells and plasmacytoid DCs (pDCs) as opposed to the MAVS pathway in mouse bone marrow-derived DCs

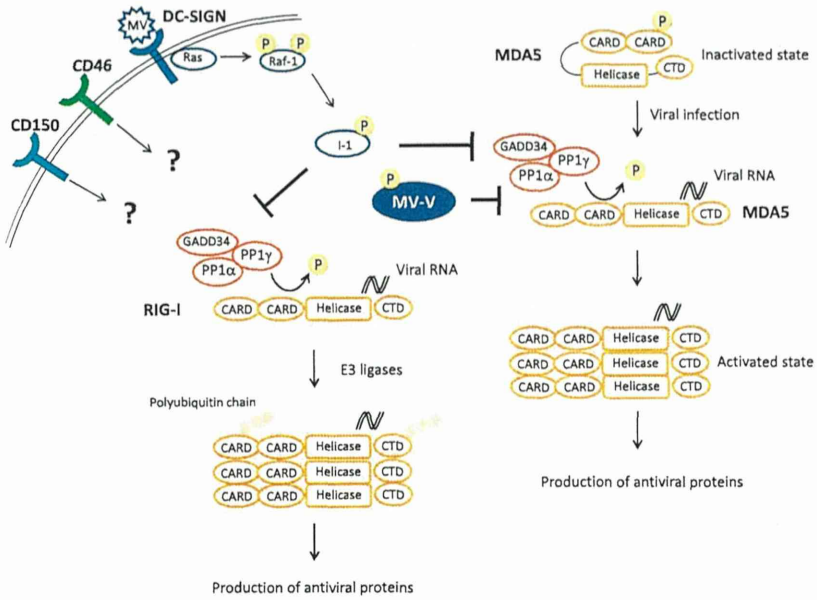

Figure 1. Measles Virus Subverts PP1-Mediated Dephosphorylation and Activation of RLRs
In response to viral infection, PP1 dephosphorylates MDA5 (right) and RIG-I (left) to induce their RNA-sensing activity, ultimately resulting in type I IFN production. However, measles virus (MV) subverts this response using two approaches. The MV-V protein acts as a competitive substrate for PP1 to block activation of MDA5. Additionally, the virus binds to the surface molecule DC-SIGN on dendritic cells and induces the activation of the Raf-1 kinase. Raf-1 phosphorylates the phosphatase inhibitor I-1, resulting in its association with GADD34-PP1 phosphatase holoenzymes and inhibition of their phosphatase activity.

(BMDCs). Although many reports have emphasized an important role of the RIG-I/MDA5-MAVS pathway in type I IFN production in MV-infected cells, such findings highlight the possibility that susceptibility to MV infection may vary depending on the type of human DC subset targeted by the virus, since each cell type activates its own unique type I IFN induction pathway in response to viral infection. MV infection studies using monkeys will be a way to test the compatibility of these studies on mouse DC subsets with human DCs.

In addition to the role of MV-V protein in targeting PP1 to prevent MDA5 activation, MV-V has also been shown to block NF-κB activation by binding to the subunit p65 RelA (Schuhmann et al., 2011) and also inhibits STAT1 signaling downstream of the interferon-α/β receptor (IFNAR). The V protein also promotes MV replication by inhibiting the MAVS pathway or possibly other unknown pathways. Therefore, MV-V appears to regulate innate immune responses in a complex fashion. Human epithelial cells express an MV entry receptor, Nectin4, and MDA5 and RIG-I participate in MV RNA recognition in this cell type. These results infer that the above IFN-regulatory mechanisms, other than the DC-SIGN-V protein axis, may participate in regulating IRF-3 and IFN induction in epithelial cells. It is notable that V protein only minimally blocks dephosphorylation of RIG-I under the conditions where dephosphorylation of MDA5 is blocked by MV-V in epithelial cells (Davis et al., 2014). Hence, it remains to be clarified whether the inhibition of MDA5 dephosphorylation by V protein is important in in vivo MV infection.

Another highlight from these studies (Mesman et al., 2014) is that the virus is regulating DCs via an interaction with molecules expressed on the cell surface. Other molecules expressed on the DC surface such as CD46 (MCP) or CD150 (SLAM) can, respectively, transmit signals that lead to autophagy or regulate B cell activation. These molecules might influence DC-specific functions. In fact, immune suppression is a characteristic feature of MV infection, which may be in part due to the failure of DC functions. Aside from antagonizing the IFN-inducing antiviral response of dsRNA, MDA5 has also been reported to be associated with secondary responses, including T lymphocyte proliferation and NK cell activation in response to poly(I:C) (Wang et al., 2010; Ebihara et al., 2010). In short, MV may interfere with the functions of DC in an early phase to further circumvent the host antiviral IFN system.

As exemplified in these studies, viruses have various strategies to escape IFN attack. The present results elucidate mechanisms by which the MAVS pathway is regulated in paramyxovirus infection. The fact that MV employs multiple strategies to inhibit RLR sensing and activation further underscores the importance of IFN as an antiviral defense.

ACKNOWLEDGMENTS

Discussions with Drs. Matsumoto, Oshiumi, Takaki, and Leong in our laboratory are gratefully acknowledged. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture and the Ministry of Health, Labor, and Welfare of Japan. Financial support by a MEXT Grant-in-Project ("the Carcinogenic Spiral") and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) are gratefully acknowledged.

REFERENCES

Childs, K.S., Andrejeva, J., Randall, R.E., and Goodbourn, S. (2009). J. Virol. *83*, 1465–1473.

Davis, M.E., Wang, M.K., Rennick, L.J., Full, F., Gableske, S., Mesman, A.W., Gringhuis, S.I., Geijtenbeek, T.B.H., Duprex, W.P., and Gack, M.U. (2014). Cell Host Microbe, in press.

de Witte, L., Abt, M., Schneider-Schaulies, S., van Kooyk, Y., and Geijtenbeek, T.B. (2006). J. Virol. *80*, 3477–3486.

Ebihara, T., Azuma, M., Oshiumi, H., Kasamatsu, J., Iwabuchi, K., Matsumoto, K., Saito, H., Taniguchi, T., Matsumoto, M., and Seya, T. (2010). J. Exp. Med. *207*, 2675–2687.

Ikegame, S., Takeda, M., Ohno, S., Nakatsu, Y., Nakanishi, Y., and Yanagi, Y. (2010). J. Virol. *84*, 372–379.

Mesman, A.W., Zijlstra-Willems, E.M., Kaptein, T.M., de Swart, R.L., Davis, M.E., Ludlow, M., Duprex, W.P., Gack, M.U., Gringhuis, S.I., and Geijtenbeek, T.B.H. (2014). Cell Host Microbe, in press.

Schuhmann, K.M., Pfaller, C.K., and Conzelmann, K.K. (2011). J. Virol. *85*, 3162–3171.

Takaki, H., Oshiumi, H., Matsumoto, M., and Seya, T. (2014). Int. J. Biochem. Cell Biol. *53C*, 329–333.

Wang, Y., Cella, M., Gilfillan, S., and Colonna, M. (2010). J. Immunol. *184*, 2751–2755.

Wies, E., Wang, M.K., Maharaj, N.P., Chen, K., Zhou, S., Finberg, R.W., and Gack, M.U. (2013). Immunity *38*, 437–449.

BJ Disease

Biochem. J. (2014) 458, 195–201 (Printed in Great Britain) doi:10.1042/BJ20131492

REVIEW ARTICLE

Beyond dsRNA: Toll-like receptor 3 signalling in RNA-induced immune responses

Megumi TATEMATSU*, Tsukasa SEYA* and Misako MATSUMOTO*[1]

*Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan

The innate immune system recognizes pathogen- and damage-associated molecular patterns using pattern-recognition receptors that activate a wide range of signalling cascades to maintain host homoeostasis against infection and inflammation. Endosomal TLR3 (Toll-like receptor 3), a type I transmembrane protein, senses RNAs derived from cells with viral infection or sterile tissue damage, leading to the induction of type I interferon and cytokine production, as well as dendritic cell maturation. It has been accepted that TLR3 recognizes perfect dsRNA, but little has been addressed experimentally with regard to the structural features of virus- or host-derived RNAs that activate TLR3. Recently, a TLR3 agonist was identified, which was a virus-derived 'structured' RNA with incomplete stem structures. Both dsRNA and structured RNA are similarly internalized through clathrin- and raftlin-dependent endocytosis and delivered to endosomal TLR3. The dsRNA uptake machinery, in addition to TLR3, is critical for extracellular viral RNA-induced immune responses. A wide spectrum of TLR3 ligand structures beyond dsRNA and their delivery systems provide new insights into the physiological role of TLR3 in virus- or host-derived RNA-induced immune responses. In the present paper, we focus on the system for extracellular recognition of RNA and its delivery to TLR3.

Key words: dendritic cell, dsRNA, endocytosis, innate immunity, structured RNA, Toll-like receptor (TLR), type I interferon, uptake receptor, viral infection.

INTRODUCTION

The immune system has developed a strategy for maintaining host homoeostasis through its interaction with environmental microbes. An array of PRRs (pattern-recognition receptors) in the innate immune system recognizes PAMPs (pathogen-associated molecular patterns) and induces anti-microbial immune responses [1]. Endosomal TLRs (Toll-like receptors) 3, 7, 8 and 9 serve as sensors of exogenous nucleic acids, whereas cytoplasmic RLRs (RIG-I-like receptors), AIM2-like receptors and DDX family members recognize intracellular viral nucleic acids [2,3]. The compartmentalization of PRRs is important for sensing both extra- and intra-cellular PAMPs and transmitting signals via distinct adaptor molecules.

Among the nucleic acid-sensing TLRs, TLR3 that recognizes dsRNA has a unique expression profile and subcellular localization [4,5]. It is expressed in immune cells, including myeloid DCs (dendritic cells) and macrophages, and in non-immune cells such as fibroblasts, epithelial cells and neurons [5–7]. TLR3 localizes to the early endosome in myeloid DCs [8], whereas macrophages, fibroblasts and some epithelial cell lines express TLR3 both on the cell surface and in the early endosome [5,9]. Although TLR3s on the cell surface participate in dsRNA recognition [5], TLR3-mediated signalling is initiated from endosomal compartments in either cell type [8].

In the case of TLR3, virus-derived dsRNA and poly(I:C) (polyriboinosinic:polyribocytidylic acid), a synthetic dsRNA, were first identified as TLR3 ligands [4,5]. dsRNA exists as a viral genome or is generated in the cytosol during replication of positive-strand RNA viruses and DNA viruses [10]. Thus TLR3 appears to sense extracellular viral dsRNA released from infected cells and activates antiviral immunity [11]. Indeed, TLR3 mediates a protective response against positive-strand RNA virus infection, including PV (poliovirus), coxsackievirus group B serotype 3 and encephalomyocarditis virus, and DNA virus infection such as herpes simplex virus 1 and murine cytomegalovirus (Table 1) [12–19]. On the other hand, detrimental effects of TLR3 in host immunity to some RNA and DNA viruses also have been demonstrated [20–23]. Notably, TLR3-mediated signalling exacerbates negative-strand RNA virus infection, in which dsRNA is barely detectable [22,23]. In addition, RNA released from damaged cells or mRNA is also recognized by TLR3 [24,25]. However, little is known about which RNA molecules or structures activate TLR3 during infection or inflammation. We identified recently a structural unit that can activate TLR3; surprisingly, this 'structured' RNA recognized by TLR3 contains an incomplete stem with bulge and internal loops, but sufficiently induces type I IFNs (interferons) and pro-inflammatory cytokines in both human and mouse cells [26]. Hence the spectrum of TLR3 ligand structures appeared to be beyond the canonical dsRNA. The results offer new insights into the physiological role of TLR3 in virus- or host-derived RNA-induced immune responses. In the present review, we focus on exRNA (extracellular RNA) recognition and signalling by TLR3.

Abbreviations: AP-1, activator protein-1; CTL, cytotoxic T-cell; DC, dendritic cell; ECD, ectodomain; exRNA, extracellular RNA; HEK, human embryonic kidney; IFN, interferon; IL, interleukin; iPSC, induced pluripotent stem cell; IRF-3, IFN regulatory factor-3; LRR, leucine-rich repeat; LRR-CT, LRR C-terminal; LRR-NT, LRR N-terminal; MDA5, melanoma differentiation-associated gene 5; NF-κB, nuclear factor κB; NK, natural killer; ODN, oligodeoxynucleotide; PAMP, pathogen-associated molecular pattern; poly(I:C), polyriboinosinic:polyribocytidylic acid; PRR, pattern-recognition receptor; PV, poliovirus; RIG-I, retinoic acid inducible gene-I; RLR, retinoic acid inducible gene-I-like receptor; TICAM-1, Toll–IL-1 receptor domain-containing adaptor molecule-1; TIR, Toll–IL-1 receptor; TLR, Toll-like receptor.

[1] To whom correspondence should be addressed (email matumoto@pop.med.hokudai.ac.jp).

Table 1 Role of TLR3 in viral infections

Viral genome	Protection	Deterioration
(+) ssRNA	Poliovirus [12,13], coxsackievirus group B serotype 3 [14] and encephalomyocarditis virus [15]	West Nile virus [20]
dsRNA	Rotavirus [16]	
dsDNA	Herpes simplex virus 1 [17,18] and murine cytomegalovirus [19]	Vaccinia virus [21]
(−) ssRNA		Influenza A virus [22] and phlebovirus [23]

Table 2 Representative TLR3 ligands identified by *in vivo* or *in vitro* experiments using reporter assay and TLR3-deficient mouse DC/macrophage stimulation

Ab, antibody; PBMC, peripheral blood mononuclear cell.

RNA ligands for TLR3	Details	*In vitro* assay	Reference(s)
Exogenous RNA			
Viral dsRNA	Reovirus genome dsRNA	Mouse DC	[4]
Viral mRNA	*In vitro* transcribed HIV gag mRNA	HEK-293/TLR3	[24]
Viral structured RNA	*In vitro* transcribed PV RNA	Mouse DC and HEK-293/TLR3	[26]
Bacterial RNA	*Escherichia coli* total RNA	HEK-293/TLR3	[73]
	dsRNA of lactic acid bacteria	Mouse DC	[74]
Endogenous RNA			
RNA from necrotic cells	RNA from necrotic neutrophils	Macrophage	[25]
	UVB-irradiated U1 RNA (small nuclear RNA)	Human PBMC	[42]
Synthetic dsRNA	Poly(I:C)	HEK-293/TLR3 and Ab inhibition	[5]
	Poly(I:C_{12}U)	Mouse DC	[75]
In vitro transcribed dsRNA	Measles virus cDNA	HEK-293/TLR3	[31]
	pFastBac-CPrME plasmid	Mouse DC and HEK-293/TLR3	[37,41]

RECOGNITION OF dsRNA BY TLR3

TLR3 recognizes viral or *in vitro* transcribed dsRNA in a sequence-independent manner and mediates downstream signalling via TICAM-1 (TIR domain-containing adaptor molecule-1; also known as TRIF) [27,28]. 5′-Triphosphorylation of dsRNA is dispensable for TLR3 recognition, differing from the dsRNA recognition mode of RIG-I (retinoic acid inducible gene-I) [29,30]. Furthermore, 2′-hydroxy groups are essential for TLR3 activation by poly(I:C), because 2′-O-methyl or 2′-fluoro modification of cytidylic acid abolishes the TLR3 activating ability of the I/C duplex [31].

TLR3 consists of an ECD (ectodomain) containing 23 LRRs (leucine-rich repeats) and the LRR-NT (LRR N-terminal) and LRR-CT (LRR C-terminal) regions, the transmembrane domain, the cytoplasmic linker region and the TIR (Toll–IL-1 receptor) domain [32]. Crystallized human TLR3 ECD is a horseshoe-shaped solenoid assembled from 23 LRRs, of which one face is largely masked by carbohydrate, whereas the other is unglycosylated [33,34]. The N-terminal histidine residues (His^{39} in LRR-NT, His^{60} in LRR1 and His^{108} in LRR3) and the C-terminal His^{539} and Asn^{541} in LRR20 of TLR3 ECD are indispensable for dsRNA binding [33–36]. The histidine residues are protonated at endosomal pH (~pH 6.0), generating an ionic attraction with the negatively charged phosphate backbone of dsRNA. Leonard et al. [37] showed that the TLR3 ECD binds as a dimer to a 40–50 bp length of dsRNA and that multiple TLR3 ECD dimers bind to long dsRNA strands. Binding affinities increase with both buffer acidity and dsRNA length. Structural analysis of the mouse TLR3 ECD–46-bp dsRNA complex revealed that dsRNA interacts with both an N- and a C-terminal-binding site on the glycan-free surface of each TLR3 ECD, which are on opposite sides of the dsRNA [38]. The ribose-phosphate backbone is the major determinant of binding, accounting for sequence-independent dsRNA binding to TLR3. In addition, the two LRR-CT regions come together, which is essential for stable receptor–ligand complex formation and facilitates the dimerization of the cytoplasmic TIR domain [39]. Indeed, a TLR3 mutant lacking LRR21 is constitutively active, probably because of ligand-independent dimer formation due to the altered configuration of the C-terminal TLR3 ECD structure [40].

Although a biochemical study showed that a dsRNA of 40–50 bp in length forms a stable complex with dimeric TLR3 ECD under acidic conditions (pH 5.5) [36], a dsRNA of >90 bp in length is required for TLR3-mediated cytokine production and DC maturation when added to mouse DCs [41]. Given that a dsRNA of >90 bp in length is required for stable complex formation with TLR3 at the pH within the early endosome (~pH 6.0–6.5), and that TLR3 localizes to the early endosome, TLR3 oligomerization in the early endosome is essential for downstream signalling.

RECOGNITION OF VIRUS- OR HOST-DERIVED RNA BY TLR3

Several reports suggest that TLR3 recognizes RNA molecules other than dsRNA (Table 2). In negative-sense RNA virus infections, such as influenza A virus and phlebovirus, which generate little dsRNA as intermediate replication products, TLR3-mediated inflammatory cytokine and chemokine production affects virus-induced pathology and host survival [22,23]. In addition, Karikó et al. [24] reported that *in vitro* transcribed HIV gag mRNA complexed with lipofectin activates TLR3. Cavassani et al. [25] also demonstrated that mouse macrophages responded to RNA from sterile necrotic neutrophils in a TLR3-dependent manner. However, which RNA molecules or structures of the virus- or host-derived RNAs activate TLR3 is unknown. A recent study showed that RNA from UVB-irradiated keratinocytes induces cytokine production in normal