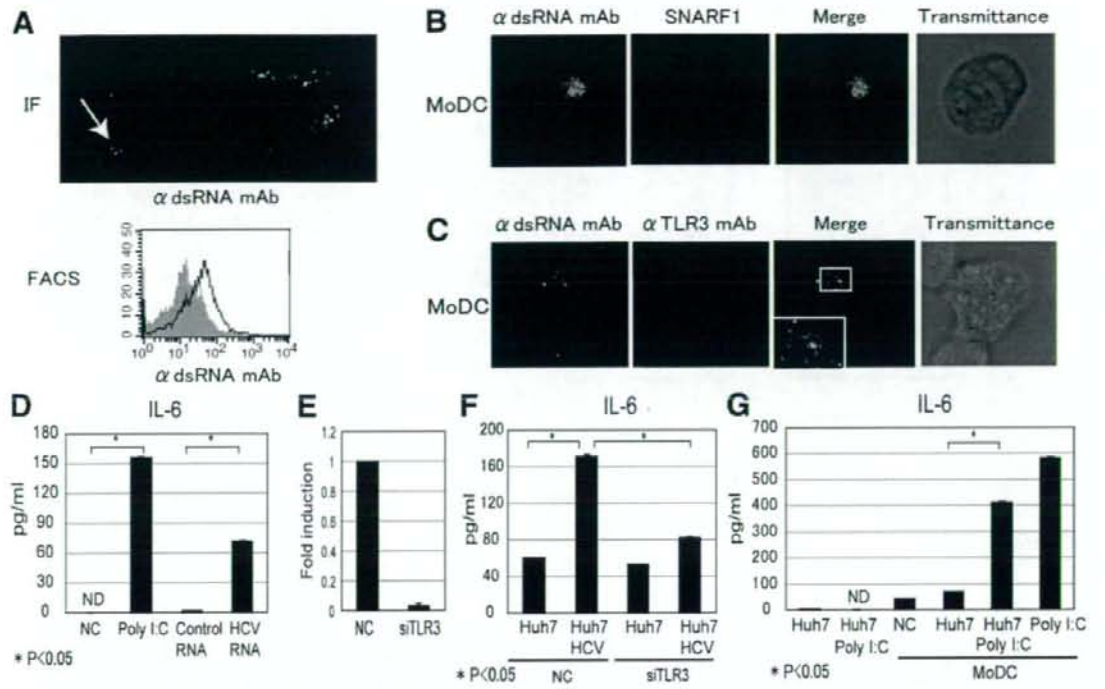

Fig. 8. TLR3 signaling by dsRNA contained in apoptotic vesicles induces MoDC maturation. (A) HCV-infected apoptotic Huh7.5.1 cells were labeled with SNARF1. dsRNA was detected in HCV-infected apoptotic cells and vesicles (arrow) by immunofluorescent assay (upper panel) and FACS using anti-dsRNA mAb (lower panel). (B,C) MoDCs were exposed to HCV-infected apoptotic cells for 4 hours and harvested for immunofluorescent assay. The MoDCs were isolated with Ficoll-Paque and stained with mAbs against dsRNA or human TLR3. (D) MoDCs were transfected with total RNA extracted from HCV-infected or noninfected Huh7.5.1 cells. After 1 day of culture, the levels of IL-6 were determined. Poly I:C was positive control. (E) The level of TLR3 messenger RNA (mRNA) was determined 1 day after siRNA electroporation. (F) Knockdown of TLR3 partially abolishes the IL-6 production by MoDCs. Data shown are means ± SD of duplicate or triplicate samples from one experiment representative of three donors. (G) Poly I:C-transfected apoptotic Huh7.5.1 cells induced MoDCs to produce IL-6.

inflammatory cytokine was a representative marker for TLR3 signal in this case, suggesting that at least HCV RNA, rather than proteins, participates in MoDC maturation.

Since siRNA knockdown of TLR3 in MoDCs was successfully executed by electroporation of MoDCs with TLR3-targeted siRNA (Fig. 8E), we tested whether the level of IL-6 was affected in the TLR3-depleted MoDCs stimulated with apoptotic vesicles containing dsRNA of HCV propagation. TLR3-depleted MoDCs retarded maturation into decreased IL-6 production (Fig. 8F). Poly I:C-transfected Huh7.5.1 apoptotic cells stimulate MoDCs to secret IL-6 (Fig. 8G). Taken together, phagocytosed HCV-infected apoptotic cells can provoke TLR3 signaling in MoDCs, which participates in MoDC maturation.

MoDCs are known to take up polyI:C, a synthetic dsRNA, which is recognized by TLR3. Therefore, MoDC maturation may be elicited by direct MoDC uptake of dsRNA produced during HCV replication. However, CD86 up-regulation was not observed on MoDCs stimulated with freeze/thaw cell lysates and sonicated apoptotic cells from HCV-infected Huh7.5.1 cells (Fig. 9A). For MoDC maturation, dsRNA was required to be wrapped in vesicles.

We next treated MoDCs with CPZ (a known inhibitor of clathrin-mediated endocytosis), BAF (a specific inhibitor of the vacuolar H^+-adenosine triphosphatase), and methyl-beta-cyclodextrin (MβCD, which depletes or sequesters membrane cholesterol, inhibiting endocytic pathways dependent on lipid rafts) to evaluate the possible involvement of endocytosis in MoDC maturation. MβCD had an inhibitory effect on MoDC maturation (Fig. 9B) and cytokine responses (data not shown) by HCV-infected apoptotic cells. Lipid rafts appeared to play some important roles in the uptake of HCV-infected apoptotic cells.

Discussion

MoDC recognizes pattern molecules of pathogen to signal the presence of microbial infection.[7] Pattern recog-

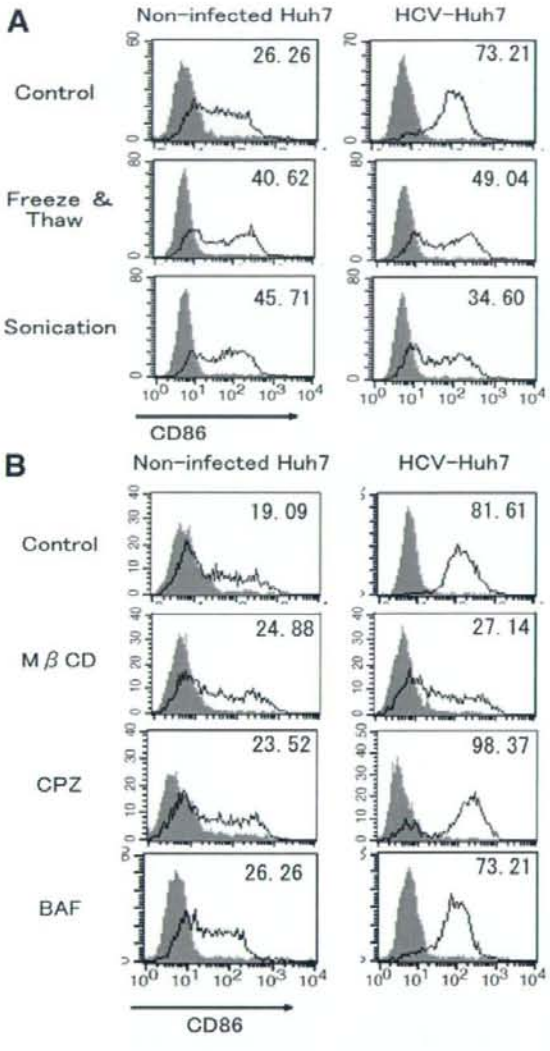

Fig. 9. Lipid raft-dependent phagocytosis of dsRNA-including apoptotic vesicles is required for MoDC maturation. (A) HCV-infected or noninfected apoptotic cells were prepared as described. The expression of CD86 was examined on MoDCs stimulated with apoptotic cell lysates prepared by freeze-thaw or sonication. (B) MoDCs were treated with methyl-beta-cyclodextrin (MβCD), CPZ, and BAF for 1 hour, followed by coculture with HCV-infected or noninfected apoptotic cells for 2 days. The MoDC CD86 expression was determined by FACS. Data from a representative of three donors are shown.

nition and antigen uptake are two central events in the activation of cellular immunity. How HCV infection elicits MoDC maturation and NK activation is the theme of this study. The following results were obtained with the JFH1 HCV strain and human MoDC. MoDCs mature via phagocytosing infected hepatocytes, but not through direct infection. MoDCs taking up HCV-infected apoptotic vesicles containing dsRNA activate T cells and NK cells. The mature MoDCs also polarized $CD4^+$ T cells into the Th1 type. Thus, HCV-infected apoptotic hepatocytes are a source of HCV antigen and PAMPs.

These *in vitro* results cast light on the mechanism of CTL and NK cell activation against HCV in patients. In the liver of patients, immature human MoDCs may phagocytose bystander hepatocytes when the cells undergo apoptosis secondary to HCV infection. The MoDCs that incorporate HCV-infected vesicles into the phagosomes are able to secrete cytokines including IFN-beta and IL-6. These MoDC responses are enabled by fusing HCV-derived dsRNA with phagosomal TLR3. Activation of the MoDC TLR3 pathway, as has been reported,[8,16] have a crucial role in development of the MoDC TLR3-mediated NK activation and CTL induction.

MoDCs express endogenous DC-SIGN, which captures pseudotype lentivirus particles expressing HCV glycoproteins E1 and E2 and may transmit HCV particles to adjacent hepatocytes.[24] Pseudotype vesicular stomatitis virus coated with chimeric E1 and E2 enters MoDCs through interaction with lectins.[22] These pseudotype HCV studies suggested that MoDCs capture, and in some cases internalize, HCV particles only when expressing E1/E2. In fact, many candidates of the HCV entry receptor have been reported and MoDCs express CD81, scavenger receptor class B type I, and DC-SIGN.[24,25] However, the actual ligand-receptor interaction in HCV-MoDC infection has not been demonstrated even in CD81 and DC-SIGN. Our results suggest that phagocytosis of HCV-infected apoptotic cells, but not direct interaction between MoDCs and HCV particles, serves as an inducer of MoDC maturation. The molecules on HCV-infected cells rather than those only in the virion may participate in induction of MoDC-mediated HCV cellular immunity.

NK cells play a role to prevent persistent HCV infection. An epidemiologic survey showed that genes encoding the weak inhibitory NK cell receptor KIR2DL3 and its human leukocyte antigen C group1 ligand are directly associated with HCV eradication in patients.[3] Since the cell-cell contact is indispensable for MoDC-mediated NK activation, soluble factors such as type I IFN and IL-15 may only have a peripheral role in the emergence of HCV-derived NK cells. We have shown that natural killer group 2, member D (NKG2D) ligands on MoDCs, which interact with NKG2D on NK cells are involved in MoDC-mediated NK cell activation against RNA virus infection and poly I:C.[9] The NKG2D/NKG2D ligand interaction was partially responsible for NK activation by MoDCs after uptake of dsRNA-containing vesicles (data

not shown). Yet the main ligand for NK activation on MoDCs is still undetermined. Searching for dsRNA-inducible NK activation ligands in MoDCs would foster identification of MoDC factors reciprocally activating NK cells.

In general, high replication of viruses results in cell death by apoptosis and necrosis. Our study on HCV suggests that apoptotic alteration occurs in HCV-infected Huh7.5.1 cells. These HCV-infected cells fostered MoDCs to produce IL-6 (Fig. 5A) and activate NK cells and T cells regardless of their apoptotic alteration by TNF-alpha and cycloheximide (data not shown). There is also evidence showing that apoptotic lesions exist in the liver of patients with HCV hepatitis by histological examination.[26] Hence, it is acceptable that MoDCs take up apoptotic hepatocytes that contain HCV antigens and dsRNA in that lesion. Schulz et al.[8] reported that TLR3 in myeloid DCs promotes cross-priming to virus-infected cells using mouse bone marrow–derived DCs and Vero cells containing polyI:C or infected with dsRNA-producing picornavirus.[8] This model study, however, regrettably involves the process of xenogeneic cell-cell interaction. Nevertheless, our present study supports their notion in the human system and offers the possibility that myeloid DC maturation is reproduced by HCV-infected hepatocytes in HCV patients.

There have been many controversial reports about whether MoDCs were infected with HCV and deficient in the allostimulatory capacity in patients with chronic HCV infection.[10] A number of HCV proteins were suggested to affect MoDC function by overexpression studies. HCV core and E1 proteins inhibit the MoDC allostimulatory activity.[27] NS3/4A is a protease that inactivates the IFN-inducing pathways by cleaving the adapter molecules of RIG-I/MDA5 and TLR3, MAVS/Cardif/IPS-1/VISA,[11] and TICAM1/TRIF,[12] respectively. However, these proteins may not be authors for myeloid DC modulation, since HCV replication in MoDCs was not detected *in vitro* and HCV replicates in MoDCs from HCV patients were at very low copy numbers, if any.[10] Although defective MoDCs were reported to appear in HCV patients,[10] this may not merely be due to the DC-HCV interaction since HCV perturbs many cells and mediators in infected lesions.

In the HCV-infected apoptotic cells, there are HCV proteins as well as HCV-derived dsRNA. Therefore, the possibility still remains that not only dsRNA but also phagocytosed HCV proteins are involved in MoDC maturation. Although what happens in HCV natural infection is unclear, our study revealed that MoDC does not mature in response to lysates of HCV-infected apoptotic cells.

Sensor proteins for dsRNA reside in the cytoplasm as well as the cell surface.[7] In MoDCs, MDA5 and RIG-I may be engaged in sensors for HCV dsRNA in HCV-infected apoptotic cells. In this case, however, the dsRNA in phagosomes must pass through the membrane to encounter cytosolic RIG-I/MDA5. Thus, the possible interpretation is that apoptotic vesicles with HCV dsRNA are incorporated into the TLR3-bearing phagosome in MoDCs (Fig. 8C).

TLR3 is the receptor for dsRNA on the endosome membrane and engaged in MoDC maturation.[18] This maturation is inhibited by BAF and chloroquine, indicating that pH changes within intracellular compartments are critical for the process.[28] Opposing to these reports, treating MoDCs with BAF (Fig. 9) or chloroquine (data not shown) results in no inhibition of MoDC maturation in our HCV-incorporated vesicle studies. One possibility deduced from the BAF test is the presence of an alternative source for viral dsRNA recognition that is independent of endosomal acidification. Lipid rafts wherein HCV-infected apoptotic cells are phagocytosed (Fig. 9), may be associated with acidification-free MoDC maturation. Our data, including colocalization of dsRNA with TLR3 and partial blocking TLR3 with siRNA also suggested that TLR3 and HCV dsRNA assemble in the same compartment. Further studies on the dsRNA recognition facility in the phagosomes as well as possible participation of MDA5 and RIG-I in MoDC-NK reciprocal activation will be needed to clarify the mechanism of HCV-mediated MoDC maturation.

Acknowledgment: We are grateful to Drs. H. Oshiumi, M. Sasai, A. Ishii, K. Funami, and A. Matsuo in our laboratory for their experimental support. We appreciate Drs. K. Shimotohno (Kyoto University, Kyoto) and Y. Matsuura (Osaka University, Osaka) for their critical discussions. Thanks are also due to Drs. Chisari (The Scripps Research Institute, La Jolla, CA) for providing the cell line.

References

1. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. Lancet 2003; 362:2095-2100.
2. Haller O, Kochs G, Weber F. The interferon response circuit: induction and suppression by pathogenic viruses. Virology 2006;344:119-130.
3. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 2004;305:872-874.
4. Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, et al. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. HEPATOLOGY 2001;33:267-276.
5. Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med 2001;194:1395-1406.

6. Bigger CB, Brasky KM, Lanford RE. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. J Virol 2001;75:7059-7066.
7. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783-801.
8. Schulz O, Diebold SS, Chen M, Naslund TI, Nolte MA, Alexopoulou L, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005;433:887-892.
9. Andrews DM, Andoniou CE, Scalzo AA, van Dommelen SL, Wallace ME, Smyth MJ, et al. Cross-talk between dendritic cells and natural killer cells in viral infection. Mol Immunol 2005;42:547-555.
10. Pachiadakis I, Pollara G, Chain BM, Naoumov NV. Is hepatitis C virus infection of dendritic cells a mechanism facilitating viral persistence? Lancet. Infect Dis 2005;5:296-304.
11. Li XD, Sun L, Seth RB, Pineda G, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci U S A 2005;102: 17717-17722.
12. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A 2005;102:2992-2997.
13. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
14. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
15. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 2007; 315:1398-1401.
16. Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, Tsujimura K, et al. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci U S A 2007;104:252-257.
17. Ebihara T, Masuda H, Akazawa T, Shingai M, Kikuta H, Ariga T, et al. NKG2D ligands are induced on human dendritic cells by TLR ligand stimulation and RNA virus infection. Int Immunol 2007;19:1145-1155.
18. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 2003;171:3154-3162.
19. Castet V, Fournier C, Soulier A, Brillet R, Coste J, Larrey D, et al. Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected in vitro. J Virol 2002;76:8189-8199.
20. Sosnovtsev SV, Prikhod'ko EA, Belliot G, Cohen JI, Green KY. Feline calicivirus replication induces apoptosis in cultured cells. Virus Res 2003; 94:1-10.
21. Prechtel AT, Turza NM, Theodoridis AA, Steinkasserer A. CD83 knockdown in monocyte-derived dendritic cells by small interfering RNA leads to a diminished T cell stimulation. J Immunol 2007;178: 5454-5464.
22. Kaimori A, Kanto T, Kwang C, Limn, Komoda Y, Oki C, et al. Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin. Virology 2004;324:74-83.
23. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526-1529.
24. Cormier EG, Durso RJ, Tsamis F, Boussemart L, Manix C, Olson WC, et al. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. Proc Natl Acad Sci U S A 2004;101:14067-14072.
25. Yamada E, Montoya M, Schuettler CG, Hickling TP, Tarr AW, Vitelli A, et al. Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets. J Gen Virol 2005;86:2507-2512.
26. Kountouras J, Zavos C, Chatzopoulos D. Apoptosis in hepatitis C. J Viral Hepat 2003;10:335-342.
27. Sarobe P, Lasarte JJ, Zabaleta A, Arribillaga L, Arina A, Melero I, et al. HCV structural proteins impair dendritic cell maturation and inhibit in vivo induction of cellular immune responses. J Virol 2003;77:10862-10871.
28. de Bouteiller O, Merck E, Hasan UA, Hubac S, Benguigui B, Trinchieri G, et al. Recognition of double-stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH. J Biol Chem 2005;280:38133-38145. 6.