Table 2. Expression of nucleic acid-sensing TLRs in DC subsets

	DC subset	TLR3	TLR7	TLR8	TLR9	References
Human	Myeloid DC					
	MoDC	þ		þ		[31–35]
	CD11c^{þ}CD1c^{þ}DC	þ		þ		[34,35,94,95]
	CD141^{þ}CLEC9A^{þ}DC	þ þ		þ		[94,95]
	Plasmacytoid DC		þ		þ	[34,35]
Mouse	Myeloid DC					
	BMDC	þ			þ	[95]
	CD8a^{þ} DC	þ þ			þ	[93,95]
	Plasmacytoid DC		þ		þ	[93,95]

MoDC, monocyte-derived immature dendritic cells; BMDC, bone marrow-derived DC.

promote CD8^{þ} T-cell cross-priming [99]. Thus, both TLR3- and IFN-a/b-mediated signalling are likely implicated in licensing DCs for the cross-priming of CD8^{þ} T cells.

In humans, Ebihara et al. [100] demonstrated the role of TLR3, expressed in myeloid DCs, in the immune response to HCV infection. The JFH1 strain of HCV does not directly infect or stimulate myeloid DCs to activate T cells and NK cells, but instead the phagocytosis of HCV-infected apoptotic cells that contain HCV-derived dsRNA and their interaction with the TLR3 pathway in myeloid DCs, plays a critical role in DC maturation and activation of T and NK cells [100]. In addition, Jongbloed et al. [94] reported that CD141^{þ} DCs are able to cross-present viral antigens from human cytomegalovirus-infected necrotic fibroblasts. Physiologically, TLR3 in a DC subset specialized for antigen presentation appears to encounter viral dsRNAs in the endosome by uptake of apoptotic or necrotic virus-infected cells and signals for cross-presentation of viral antigens. Furthermore, a dominant-negative TLR3 allele was found in children with herpes simplex virus 1 (HSV-1) encephalitis [101]. TLR3 is expressed in the central nervous system, where it is required to control HSV-1. Interestingly, recent paper demonstrated that mouse CD8a^{þ} DCs and human CD141^{þ} DCs are major producers of IFN-λ in response to poly(I:C), which depends on TLR3 [102]. Thus, TLR3 plays a role in the antiviral response, dependent on the viral genome structure, the route of virus entry into cells, the TLR3-expressing cell type that encounters viral dsRNA, and the properties of the host anti-viral effector functions.

Application of the TLR3 ligand to adjuvant vaccine therapy

Selective expression of TLR3 in myeloid DCs, especially human CD141^{þ} DCs and mouse CD8a^{þ} DC subsets, is the advantage in employing TLR3 ligands as adjuvant. In addition to the TLR3-dependent CTL activation described above, DC-mediated NK cell activation is also important for the adjuvancy of TLR3 ligands. Akazawa et al. [103] showed that the TLR3–TICAM-1 pathway is essential for poly(I:C)-induced NK-cell-mediated tumour regression in a syngeneic mouse tumour implant model. Remarkably, production of IFN-a is not impaired in TICAM-1 $^{/}$ mice compared with wild-type mice after in vivo poly(I:C) injection or in vitro bone marrow-derived DC (BMDC) stimulation, whereas IL-12 production is completely dependent on TICAM-1, consistent with other reports [22,104]. Furthermore, NK cell activation requires cell–cell contact with BMDCs preactivated by poly(I:C) but not IFN-a or IL-12. Thus, the TLR3–TICAM-1 pathway in myeloid DCs facilitates the DC–NK cell interaction following NK cell activation. TICAM-1–IRF3-dependent expression of a novel molecule, namely IRF-3-dependent NK-activating molecule (INAM), in myeloid DCs is required for NK activation [104]. Poly(I:C)-induced MDA5-dependent myeloid DC activation is also implicated in NK cell activation [105,106].

However, several issues remain unresolved including a suitable transport system for TLR3 ligands. Poly(I:C) injected intraperitoneally in mice activates both TLR3 and MDA5, indicating that extracellular poly(I:C) is delivered to endosomal TLR3 and further to cytosolic MDA5 in murine cells. A recent study demonstrated that CD14 enhances poly(I:C)-mediated TLR3 activation in bone marrow-derived macrophages by directly binding to poly(I:C) and mediating cellular uptake of poly(I:C) [107]. The internalized poly(I:C) then colocalizes with CD14 and TLR3. Since the extracellular domain of CD14 consists of LRRs [108], CD14 may associate with TLR3 and transfer poly(I:C) to TLR3 in macrophage endosomes. In the case of CD14-negative myeloid DCs, extracellular dsRNA must be internalized with the putative uptake receptor. Indeed, it has been demonstrated by our group and others that poly(I:C) is internalized into human monocyte-derived immature DCs and mouse BMDCs via clathrin-dependent endocytosis, and B- and C-type oligodeoxynucleotides share the uptake receptor with poly(I:C) [109]. Notably, among various synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in myeloid DCs. By contrast, in vitro-transcribed dsRNAs of various lengths (50–1000 bp) cannot be internalized into myeloid DCs [110]. Thus, uptake of TLR3 ligands largely depends on the dsRNA structure recognized by the uptake receptor expressed on myeloid DCs.

The dsRNA structure and the targeting approach of dsRNA to the endosomal TLR3 in the appropriate DC subset, are important factors involved in generating innate and adaptive immune responses by TLR3 ligands. Gowen et al. [111] showed that poly(I:C_{12}U) induces IFN-b in a TLR3-dependent and MDA5-independent manner, and exhibits protective anti-viral effects in mice. Identification of the putative dsRNA uptake receptor is crucial for analysing the intracellular transport of dsRNA. Furthermore, clarification of the differences between the RIG-I/MDA5-mediated and TLR3–TICAM-1-mediated signalling pathways is important for assessment of dsRNA-induced immune responses.

Concluding remarks

The protective role of TLR3 in virus infection is now becoming clear from experiments using an infectious mouse model or TLR3-transgenic mice. Since both CVB3 and poliovirus belong to the Picornaviridae family, along with encephalomyocarditis virus that is recognized by MDA5, distinct virus properties rather than virus genome structure appear to determine which RNA sensors act in antiviral defense in host cells. The molecular mechanism behind the anti-viral function of TLR3 in vivo and the identification of TLR3–TICAM-1-mediated signalling cascades distinct from those of RIG-I/MDA5, are important factors for understanding the role of RNA-sensors in the host defense system. In addition, characterization of a new myeloid DC subset that expresses a high level of TLR3 and has a high capacity to present antigen from apoptotic and necrotic cells after TLR3 activation, may provide insight into the role of TLR3 in the activation of NK cells and CTLs in viral infection. This, in turn, may advance the development of TLR3-related vaccine adjuvants effective against tumours and/or infectious diseases.

REFERENCES

1. Muller U, Steinhoff U, Reis LFL, *et al.* Functional role of type I and type II interferons in antiviral defense. *Science* 1994; 264: 1918–1921.
2. Vilcek J. Fifty years of interferon research: aiming at a moving target. *Immunity* 2006; 25: 343–348.
3. Garcia-Sastre A, Biron CA. Type I interferons and the virus-host relationship: a lesson in Détente. *Science* 2006; 312: 879–882.
4. Clemens MJ. PKR-a protein kinase regulated by double-stranded RNA. *International Journal of Biochemistry and Cell Biology* 1997; 29: 945–949.
5. Samuel CE. Antiviral actions of interferon, interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* 1991; 183: 1–11.
6. 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. *Nature Immunology* 2004; 5: 730–737.
7. 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. *Journal of Immunology* 2005; 175: 2851–2858.
8. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124: 783–801.
9. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. RNA and activation of NF-kB by Toll-like receptor 3. *Nature* 2001; 413: 732–738.
10. Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Bio-*

chemical and Biophysical Research Communications 2002; 239: 1364–1369.
11. Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Advanced Drug Delivery Reviews* 2008; 60: 805–812.
12. Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-stranded RNA is produced by positive-stranded RNA viruses and DNA viruses but not in detectable amounts by negative-stranded RNA viruses. *Journal of Virology* 2006; 80: 5059–5064.
13. Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; 408: 740–745.
14. 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: 1526–1529.
15. Diebold SS, Kaisho T, Hemmi H, Akira S, Sousa RC. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004; 303: 1529–1531.
16. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* 2007; 315: 1398–1401.
17. Hornung V, Ellegast J, Kim S, *et al.* 5'-Triphosphate RNA is ligand for RIG-I. *Science* 2006; 314: 994–997.
18. Pichlmair A, Schulz O, Tan CP, *et al.* RIG-I-mediated anti-viral responses to single-stranded RNA bearing 5'-phosphates. *Science* 2006; 314: 997–1001.
19. Schlee M, Roth A, Hornung V, *et al.* Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 2009; 31: 25–34.
20. Schmidt A, Schwerd T, Hamm W, *et al.* 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Science of the United States of America* 2009; 106: 12067–12072.
21. Rehwinkel J, Tan CP, Goubau D, *et al.* RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell* 2010; 140: 397–408.
22. Kato H, Takeuchi O, Mikamo-Satoh E, *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *Journal of Experimental Medicine* 2008; 205: 1601–1610.
23. Kato H, Sato S, Yoneyama M, *et al.* Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 2005; 23: 19–28.
24. 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: 101–105.
25. 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. *Proceedings of the National Academy of Science of the United States of America* 2006; 103: 8459–8464.
26. Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M Jr. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 2008; 454: 523–527.
27. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JFA. A family of human receptors structurally related to Drosophila Toll. *Proceedings of the National Academy of Science of the United States of America* ; 95: 588–593.
28. Prehaud C, Megret F, Lafage M, Lafon M. Virus infection switches TLR-3-positive human neurons to become strong producers of beta interferon. *Journal of Virology* 2005; 79: 12893–12904.
29. Farina C, Krumbholz M, Giese T, Hartmann G, Aloisi F, Meinl E. Preferential expression and function of Toll-like receptor 3 in human astrocytes. *Journal of Neuroimmunology* 2005; 159: 12–19.
30. Town T, Jeng D, Alexopoulou L, Tan J, Flavell RA. Microglia recognize double-stranded RNA via TLR3. *Journal of Immunology* 2006; 176: 3804–3812.
31. Muzio M, Bosisio D, Polentarutti N, *et al.* Differential expression and regulation of Toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *Journal of Immunology* 2000; 64: 5998–6004.
32. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. *Journal of Immunology* 2001; 166: 249–254.
33. Kadowaki M, Ho S, Antonenko S, *et al.* Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *Journal of Experimental Medicine* 2001; 194: 863–870.
34. Hornung V, Rothenfusser S, Britisch S, *et al.* Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *Journal of Immunology* 2002; 168: 4531–4537.
35. Matsumoto M, Funami K, Tanabe M, *et al.* Subcellular localization of Toll-like receptor 3 in human dendritic cells. *Journal of Immunology* 2003; 171: 3154–3162.
36. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infection and Immunity* 2000; 68: 7010–7017.
37. Niimi K, Asano Y, Shiraishi T, *et al.* TLR3-mediated synthesis and release of eotaxin-1/CCL11 from human bronchial smooth muscle cells stimulated with double-stranded RNA. *Journal of Immunology* 2007; 178: 489–495.
38. Nakamura M, Funami K, Komori A, *et al.* Increased expression of Toll-like receptor 3 in intrahepatic bilinary epithelial cells at sites of ductular reaction in diseased livers. *Hepatology International* 2008; 2: 222–230.
39. Funami K, Sasai M, Ohba Y, Oshiumi H, Seya T, Matsumoto M. Spatiotemporal mobilization of Toll-IL-1 receptor domain-containing adaptor molecule 1 in response to dsRNA. *Journal of Immunology* 2007; 179: 6867–6872.
40. Funami K, Matsumoto M, Oshiumi H, Akazawa T, Yamamoto A, Seya T. The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. *International Immunology* 2004; 16: 1143–1154.
41. Nishiya T, Kajita E, Miwa S, DeFranco A. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *Journal of Biological Chemistry* 2005; 280: 37107–37117.
42. Miettinen M, Sareneva T, Julkunen I, Matikainen S. IFNs activate toll-like receptor gene expression in viral infections. *Genes and Immunity* 2001; 2: 349–355.
43. Heinz S, Haehnel V, Karaghiosoff M, *et al.* Species-specific regulation of Toll-like receptor 3 genes in men and mice. *Journal of Biological Chemistry* 2003; 24: 21502–21509.
44. Tanabe M, Taniguchi M, Takeuchi K, *et al.* Mechanism of up-regulation of human Toll-like receptor (TLR) 3 secondary to infection of measles virus attenuated strains. *Biochemical and Biophysical Research Communications* 2003; 311: 39–48.
45. Gay NJ, Gangloff M, Weber ANR. Toll-like receptors as molecular switches. *Nature Reviews. Immunology* 2006; 6: 693–698.

Rev. Med. Virol. 2011; 21: 67–77.
DOI: 10.1002/rmv

46. Bell JK, Mullen GED, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends in Immunology* 2003; 24: 528–533.
47. Choe J, Kelker MS, Wilson IA. Crystal structure of human Toll-like receptor 3 (TLR3) ectodomain. *Science* 2005; 309: 581–585.
48. Bell JK, Botos I, Hall PR, *et al.* The molecular structure of the Toll-like receptor 3 ligand-binding domain. *Proceedings of the National Academy of Science of the United States of America* 2005; 102: 10976–10980.
49. Bell JK, Askins J, Hall PR, Davies DR, Segal DM. The dsRNA binding site of human Toll-like receptor 3. *Proceedings of the National Academy of Science of the United States of America* 2006; 103: 8792–8797.
50. Fukuda K, Watanabe T, Tokisue T, *et al.* Modulation of double-stranded RNA recognition by the N-terminal histidine-rich region of the human Toll-like receptor 3. *Journal of Biological Chemistry* 2008; 283: 22787–22794.
51. Pirher N, Ivicak K, Pohar J, Bencina M, Jerala R. A second binding site for double-stranded RNA in TLR3 and consequences for interferon activation. *Nature Structural and Molecular Biology* 2008; 15: 761–763.
52. Leonard JN, Ghirlando R, Askins J, *et al.* The TLR3 signaling complex forms by cooperative receptor dimerization. *Proceedings of the National Academy of Science of the United States of America* 2008; 105: 258–263.
53. Liu L, Botos I, Wang Y, *et al.* Structural basis of Toll-like receptor 3 signaling with double-stranded RNA. *Science* 2008; 320: 379–381.
54. Rana TM. Illuminating the silence: understanding the structure and function of small RNAs. *Nature Reviews. Molecular Cell Biology* 2007; 8: 23–36.
55. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-b induction. *Nature Immunology* 2003; 4: 161–167.
56. Yamamoto M, Sato S, Hemmi H, *et al.* Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 2003; 301: 640–643.
57. 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 Journal* 2006; 25: 3335–3346.
58. Sarker SN, Peters K, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nature Structural and Molecular Biology* 2004; 11: 1060–1067.
59. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-b. *Journal of Biological Chemistry* 2003; 278: 49751–49762.
60. Fitzgerald KA, Rowe DC, Barnes BJ, *et al.* LPS-TLR4 signaling to IRF-3/7 and NF-kB involves the toll adapters TRAM and TRIF. *Journal of Experimental Medicine* 2003; 198: 1043–1055.
61. Funami K, Sasai M, Oshiumi H, Seya T, Matsumoto M. Homo-oligomerization is essential for Toll/IL-1 receptor domain-containing adaptor molecule-1 mediated NF-kB and IRF-3 activation. *Journal of Biological Chemistry* 2008; 283: 18283–18291.
62. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 2003; 300: 1148–1151.
63. Fitzgerald KA, McWhirter SM, Faia KL, *et al.* IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nature Immunology* 2003; 4: 491–496.
64. Sato S, Sugiyama M, Yamamoto M, *et al.* Toll/IL-1 receptor domain-containing adaptor-inducing IFN-b (TRIF) associates with TNFR-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kB and IFN-regulatory factor 3, in the Toll-like receptor signaling. *Journal of Immunology* 2003; 171: 4304–4310.
65. Sasai M, Tatematsu M, Oshiumi H, *et al.* Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the Toll-like receptor 3/4 pathway. *Molecular Immunology* 2010; 47: 1283–1291.
66. Hacker H, Redecke V, Blagoev B, *et al.* Specificity in Toll-like receptor signaling through distinct effector functions of TRAF3 and TRAF6. *Nature* 2006; 439: 204–207.
67. Oganesyan G, Saha SK, Guo B, *et al.* Critical role of TRAF3 in the Toll-like receptor-dependent and –independent antiviral response. *Nature* 2006; 439: 208–211.
68. Sasai M, Oshiumi H, Matsumoto M, *et al.* Cutting edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *Journal of Immunology* 2005; 174: 27–30.
69. Tatematsu M, Ishii A, Oshiumi H, *et al.* A molecular mechanism for Toll/IL-1 receptor domain-containing adaptor molecule-1-mediated IRF-3 activation. *Journal of Biological Chemistry* 2010; 285: 20128–20136.
70. Meylan E, Burns K, Hofmann K, *et al.* RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nature Immunology* 2004; 5: 503–507.
71. Han KJ, Su X, Xu LG, Bin LH, Zhang J, Shu HB. Mechanisms of the TRIF-induced interferon-stimulated response element and NF-kappaB activation and apoptosis pathways. *Journal of Biological Chemistry* 2004; 279: 15652–15661.
72. Kaiser WJ, Offermann MK. Apoptosis induced by the Toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. *Journal of Immunology* 2005; 174: 4942–4952.
73. Gohda J, Matsumura T, Inoue J. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not Toll/IL-1 receptor domain-containing adaptor-inducing IFN-b (TRIF)-dependent pathway in TLR signaling. *Journal of Immunology* 2004; 173: 2913–2917.
74. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie A. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nature Immunology* 2006; 7: 1074–1081.
75. Kayagaki N, Phung Q, Chan S, *et al.* DUBA: a deubiquitinase that regulates type I interferon production. *Science* 2007; 318: 1628–1632.
76. Boone DL, Turer EE, Lee EG, *et al.* The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nature Immunology* 2004; 5: 1052–1060.
77. Edelman KH, Richardson-Burns S, Alexopoulou L, Tyer KL, Flavell RA, Oldstone MBA. Does Toll-like receptor 3 play a biological role in virus infections? *Virology* 2004; 322: 231–238.
78. Hardarson HS, Baker JS, Yang Z, *et al.* Toll-like receptor 3 is an essential component of the innate stress response in virus-induced cardiac injury. *American Journal of Physiology. Heart and Circulatory Physiology* 2007; 292: 251–258.

79. Abe Y, Nagata N, Sata T, Takeuchi O, Akira S, Koike S. TLR3-TRIF pathway is important for type I IFN responses in poliovirus infection. *Proceedings of 57th Annual Meeting of the Japanese Society for Virology* 2009; 234.
80. Oshiumi H, Matsumoto M, Seya T. TICAM-1/TRIF, a TLR3 adaptor, is essential for protection against poliovirus infection. *International Immunology* 2010 22(Suppl. 1) i19. (abstract).
81. Racaniello VR. Picornaviridae: the viruses and their replication. In Fields Virology, 5th edn, Knipe DM, Howly PM (eds). Lippincott Williams & Wilkins: Philadelphia, 2007; 795–838.
82. Negishi H, Osawa T, Ogami K, *et al.* A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. *Proceedings of the National Academy of Science of the United States of America* 2008; 105: 20446–20451.
83. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nature Medicine* 2004; 10: 1366–1373.
84. Daffis S, Samuel MA, Suthar MS, Gale M Jr, Diamond MS. Toll-like receptor 3 has a protective role against West Nile virus infection. *Journal of Virology* 2008; 82: 10349–10358.
85. Guillot L, Goffic RL, Bloch S, *et al.* Involvement o Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *Journal of Biological Chemistry* 2005; 280: 5571–5580.
86. Rudd BD, Burstein E, Duckett CS, Li X, Lukacs NW. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. *Journal of Virology* 2005; 79: 3350–3357.
87. Rudd BD, Smit JJ, Flavell RA, *et al.* Deletion of TLR3 alters the pulmonary immune environment and mucs production during respiratory syncytial virus infection. *Journal of Immunology* 2006; 176: 1937–1942 .
88. Goffic RL, Baalloy V, Lagranderie M, *et al.* Detrimental contribution of the Toll-like receptor (TLR) 3 to influenza A virus-induced acute pneumonia. *PLoS Pathogens* 2006; 2: 526–535.
89. Gowen BB, Hoopes JD, Wong M-H, *et al.* TLR3 deletion limits mortality and disease severity due to phlebovirus infection. *Journal of Immunology* 2006; 177: 6301–6307.
90. Tabeta K, Georgel P, Janssen E, *et al.* Toll-like receptor 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proceedings of the National Academy of Science of the United States of America* 2004; 101: 3516–3521.
91. Heath WR, Belz GT, Behrens GM, *et al.* Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunology Reviews* 2004; 199: 9–26.
92. Shen L, Lock KL. Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Current Opinion in Immunology* 2006; 18: 85–91.
93. Edwards AD, Diebold SS, Slack EM, *et al.* Toll-like receptor expression in murine DC subsets: lack of CD8 aþ DC correlates with unresponsiveness to imidazoquinolines. *European Journal of Immunology* 2003; 33: 827–833.
94. Jongbloed SL, Kassianos AJ, McDonald KJ, *et al.* Human CD141þ (BDCA-3)þ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *Journal of Experimental Medicine* 2010; 207: 1247–1260.
95. Poulin LF, Salio M, Griessinger E, *et al.* Characterization of human DNGR-1þ BDCA3þ leukocytes as putative equivalents of mouse CD8aþ dendritic cells. *Journal of Experimental Medicine* 2010; 207: 1261–1271.
96. Bachem A, Güttler S, Hartung E, *et al.* Superior antigen cross-presentation and XCR1 expression define human CD11cþ CD141þ cells as homologues of mouse CD8þ dendritic cells. *Journal of Experimental Medicine* 2010; 207: 1273–1281.
97. Crozat K, Guiton R, Contreras V, *et al.* The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8aþ dendritic cells. *Journal of Experimental Medicine* 2010; 207: 1283–1292.
98. Schultz O, Diebold SS, Chen M, *et al.* Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 2005; 433: 887–892.
99. Bon AL, Etchart N, Rossmann C, *et al.* Cross-priming of CD8þ T cells stimulated by virus-induced type I interferon. *Nature Immunology* 2003; 4: 1009–1015.
100. Ebihara T, Shingai M, Matsumoto M, Wakita T, Seya T. Hepatitis C virus (HCV)-infected apoptotic cells extrinsically modulate dendritic cell function to activate T cells and NK cells. *Hepatology* 2008; 48: 48–58.
101. Zhang S-Y, Jouanguy E, Ugolini S, *et al.* TLR3 deficiency in patients with Herpes Simplex encephalitis. *Science* 2007; 317: 1522–1527.
102. Lauterbach H, Bathke B, Gilles S, *et al.* Mouse CD8aþ DCs and human BDCA3þ DCs are major producers of IFN-λ in response to poly(I:C). *Journal of Experimental Medicine* 2010; 207: 2703–2717.
103. Akazawa T, Ebihara T, Okuno M, *et al.* Antitumor NK activation induced by the TLR3–TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proceedings of the National Academy of Science of the United States of America* 2007; 104: 252–257.
104. 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. *Journal of Experimental Medicine* 2010; 207: 2675–2687.
105. Miyake T, Kumagai Y, Kato H, *et al.* Poly I:C-induced activation of NK cells by CD8 alphaþ dendritic cells via the IPS-1 and TRIF-dependent pathways. *Journal of Immunology* 2009; 183: 2522–2528.
106. 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. *Journal of Experimental Medicine* 2009; 206: 2967–2976.
107. Lee HKS, Dunzendorfer K, Soldau K, Tobias PS. 2006; Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. *Immunity* 24: 153–163.
108. Kim J-I, Lee CJ, Jin MS, *et al.* Crystal structure of CD14 and its implications for lipopolysaccharide signaling. *Journal of Biological Chemistry* 2005; 280: 11347–11351.
109. Itoh K, Watanabe A, Funami K, Seya T, Matsumoto M. The clathrin-mediated endocytic pathway participates in dsRNA-induced IFN-b production. *Journal of Immunology* 2008; 181: 5522–5529.
110. Okahira S, Nishikawa F, Nishikawa S, Akazawa T, Seya T, Matsumoto M. Interferon-b induction through Toll-like receptor 3 depends on double-stranded RNA structure. *DNA and Cell Biology* 2005; 24: 614–623.
111. Gowen BB, Wong M-H, Jung K-H, *et al.* TLR3 is essential for induction of protective immunity against Punta Toro virus infection by the double-stranded RNA (dsRNA), poly(I:C12U), but not poly(I:C): differential recognition of synthetic dsRNA molecules. *Journal of Immunology* 2007; 178: 5200–5208.

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Natural Killer Cell Activation Secondary to Innate Pattern Sensing

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Key Words
Natural killer cell activation · Dendritic cell · Toll-like receptor 3 · TICAM-1 · MyD88

Abstract
Recent progress in understanding the outcomes of pattern-recognition by myeloid dendritic cells (mDC) allows us to delineate the pathways driving natural killer (NK) cell activation. Mouse mDC mature in response to microbial patterns and are converted to an NK cell-activating phenotype. The MyD88 pathway, the Toll/IL-1 receptor homology domain-containing adaptor molecule (TICAM)-1 (TRIF) pathway, and the interferon (IFN)-β promoter stimulator 1 (IPS-1) pathway in mDC participate in driving NK activation, as shown by analyses in knockout mice. Studies using synthetic compounds for Toll-like receptors/RIG-I-like receptors have demonstrated that mDC-NK cell contact induces NK cell activation without the participation of cytokines in mice. In vivo bone marrow transplantation analysis revealed that the IPS-1 pathway in nonmyeloid cells and the TICAM-1 pathway in mDC are crucial for dsRNA-mediated in vivo NK activation. These results infer the presence of cytokine-dependent and cytokine-independent modes of NK activation in conjunction with innate immune activation. Here, we focus on the IFN-inducing pathways and mDC-NK contact-induced NK activation and discuss the reported various NK activation modes.

Introduction

Natural killer (NK) cells have the ability to kill certain tumor cells and infected cells [1]. A number of activating and inhibitory receptors have been implicated in NK cell recognition and elimination of target cells [2]. In addition, NK cell effector functions are induced or potentiated through recognition of microbial products by innate pattern recognition receptors (PRRs) that are expressed in various cell types, including myeloid cells and NK cells. In this way, dendritic cells may induce cytokines, such as interferon (IFN)-γ, and potentiate cytotoxicity by NK cells [3]. In mice, myeloid cells stimulate NK cells through cell-cell contact and with soluble mediators [4, 5]. Many factors including cytokines and molecules supporting direct contact by immune-related cells are reported to participate in NK activation [2–5]. Myeloid dendritic cells (mDC) and macrophages (Mf) often serve as a source of such activating factors as IL-12p70, IL-18, IL-15, and type I IFN.

It is well known that type I IFN activates NK cells. However, immature mDC only subfunctionally produce type I IFN and, consequently, IFN-dependent NK activation factors are maintained at basal levels through the IFN-α receptor (IFNAR) pathway in mice [6]. Infection or inflammation stimulates additional factors that render NK activation by mDC feasible [4, 5]. Such factors that induce mDC maturation largely belong to a class of ex-

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ogenous or endogenous pattern molecules designated pathogen-associated molecular patterns (PAMP)/damage-associated molecular patterns (DAMP) [7, 8]. Research into signaling pathways in the innate immune system has indicated that PAMP and DAMP act on PRRs in mDC/Mf and drive NK activation [4, 5, 7, 8]. In addition, membrane molecules upregulated on the surface of mDC participate in NK activation in a process known as mDC-NK contact-mediated NK activation [4, 5]. In this case, mature mDC and NK cells must be recruited to local lymph nodes, and their interactions lead to the emergence of effector NK cells in the periphery. However, it is unknown whether activation of NK cells is totally dependent on IFN or just shares the PRR pathways with IFN induction to upregulate other NK-activating molecules. Furthermore, it remains undetermined what microenvironment mDC require for maturation along with NK activation and what effectors mDC stimulate via the PRR pathways to participate in enhancing NK activity.

Recent progress in the innate pattern-sensing system suggests that mDC pattern recognition is a major event in driving mDC to an NK-activating phenotype [9–11]. These results add new insight into the currently accepted theory that the balance between a number of activating receptors and inhibitory receptors and their activation states are critical for NK activation [2, 3, 12, 13]. Insight into the mechanism behind NK cell activation may be gained via analysis of the molecular mechanisms by which PAMP/DAMP activate the immune system and, in particular, mDC. NK cells have the capacity to induce memory-like responses in a way comparable to T lymphocytes [14], and some subsets are specialized to produce the Th17 cytokine IL-22 [15] although their features are not always comparable to NK cells. These unique features of NK cells may be associated with mDC factors that drive NK activation, including the combination of stimuli required for PRR and the cytokines that act in conjunction with inhibitory/activating ligands on NK receptors [16]. This review collates recent advances in the innate molecules and pathways related to mDC-mediated NK activation.

Direct or Secondary Activation of NK Cells by Microbial Patterns

DC/Mf as well as stromal cells express a variety of PRRs. In infection, these non-NK cell-derived mediators play a role in NK cell responses to pathogens [17]. Activation of these cells in response to PAMP can also lead to indirect NK activation which is mediated by affected accessory cells with altered membrane-associated molecules [16, 17]. Both soluble factors and membrane molecules join NK cell activation. Several reviews mention the mode of direct and accessory cell-derived (secondary) NK activation [2, 8, 16]. We just summarized this issue to facilitate the introduction of mDC/Mf-mediated NK cell activation.

Pathogen molecules often interact with NK receptors. Examples of direct pathogen interaction with NK cells have been demonstrated in mouse cytomegalovirus, whose m157 molecule interacts with Ly49H, an NK cell-activating/inhibitory receptor [18]. Influenza virus hemaglutinins bind the NKp46 of human NK cells [19]. Besides these NK receptor-interacting molecules, several kinds of bacteria/viruses are known to directly activate NK cells by PRR stimulation. Examples of microbial ligands for Toll-like receptors (TLRs) present in the NK cell membrane are as follows. Measles virus H protein interacts with TLR2 [20]. Mycobacteria muramyl dipeptides activate TLR2 residing on the NK cell membrane [21]. *Plasmodium falciparum* has an unidentified factor that interacts with TLR2/4 [22]. Some leishmania species have a lipophosphoglycan to bind to TLR2 [23]. Pam2 lipopeptides of a variety of bacteria serve as TLR2 ligands [24]. These factors also interact with mDC/Mf TLRs. Which TLRs in NK cells or accessory cells are more important for triggering NK cell activation in vivo should be an issue to be clarified.

Overview of the mDC Pattern Sensing System

mDC, which comprise many subsets [including bone marrow-derived DC (BMDC) and CD8α+ DC], possess subset-specific pattern-recognition systems. TLR, NOD-like receptors, and RIG-I-like receptors (RLR) are representative PRRs. The PRR repertoire in mDC has been described previously [17]. Two adaptors, i.e. MyD88 and TICAM-1 (TRIF), critically determine the signal pathways for TLRs, whereas interferon-β promoter stimulator 1 (IPS-1) (Cardif, MAVS, VISA) is the only adaptor that governs MDA5/RIG-I signaling (fig. 1). These adaptors are engaged in type I IFN induction and NK activation via partly overlapped but distinct pathways in a cell type-specific manner. Here, we summarize the signal pathways for TLR and RLR relevant to NK activation.

Signaling Pathways That Operate through the MyD88 Adaptor

MyD88 is the most common adaptor in the TLR and interleukin (IL)-1R signaling pathways [25, 26]. With the

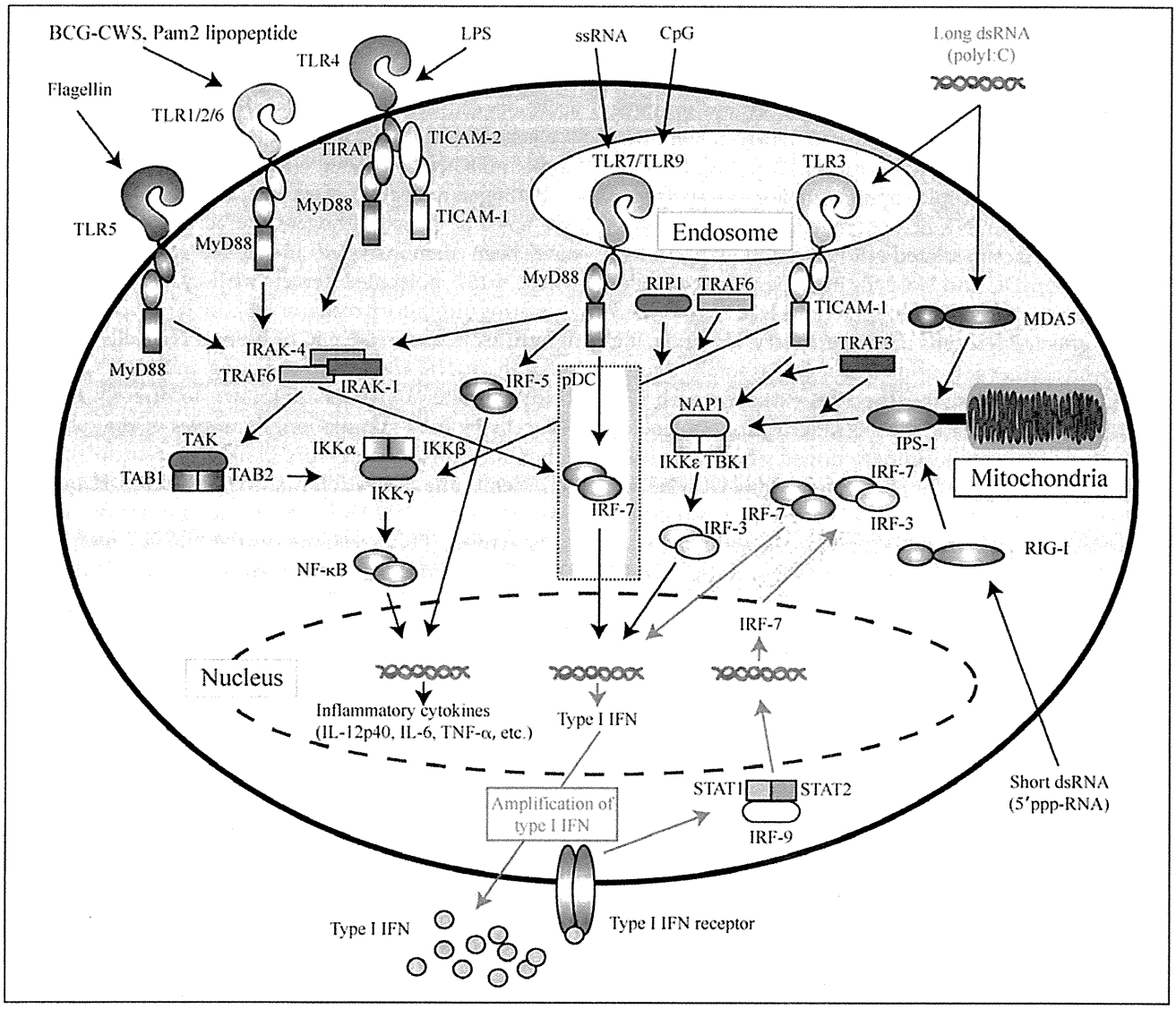

Fig. 1. TLR pattern-sensing system and cytoplasmic dsRNA recognition pathways. The TLR and RLR pattern-sensing systems involved in type I IFN induction and NK cell activation are depicted in a single cell model. pDC has a unique MyD88 pathway which is highlighted in the open square. All pathways except this MyD88 pathway function in BMDC of mouse and monocyte-derived DC of humans. Mouse spleen DC and human BDCA1/3-positive DC have weak potential for recognition of cytoplasmic dsRNA, reflecting a relatively immature feature of DC. Once TLR3 or TLR4 is stimulated, it transiently recruits TICAM-1 to form multimers. Subsequently, liberated TICAM-1 form a molecular complex named 'speckle' which serves as a nest for IRF-3/7 activation. In contrast, IPS-1 binds the outer membrane of mitochondria and forms an IRF-3/7-activating complex on the membrane. Once type I IFN is liberated, type I IFN production is amplified via the IFNAR pathway. NK cell activation occurs not only by soluble factors but also membrane proteins induced secondary to IRF-3 activation.

exception of TLR3, all TLRs in mice and humans can couple with MyD88. Only the MyD88 in plasmacytoid DC (pDC) is linked to IFN-induced signaling [27]. In pDC, TLR7/9 recruit MyD88 and the IRAK4/IRAK1 complex, allowing the phosphorylation of IRAK1 [28]. Activated IRAK1 forms a molecular complex with TRAF6/TRAF3/Osteopontin and IKKα. After forming a complex with MyD88, activated IKKα activates nearby IFN regulatory factor (IRF)-7 which, in turn, is dimerized and translocated to the nucleus [29]. The IRF-7 di-

mer then initiates transcription of the IFN-α gene. Once IFN-α is liberated, the IFNAR on the same cell or on other DC is activated to amplify the production of IFN-α [6, 28, 29]. MyD88 also recruits IRF-5 which induces inflammatory cytokines, IL-6, IL-12p40, and tumor necrosis factor (TNF)-α but is minimally associated with type I IFN induction according to knockout (KO) mouse studies [30] (fig. 1).

MyD88-deficient mice show abolished responses to IL-1β and IL-18 and to the ligands for TLR2, TLR5, TLR7, and TLR9 [17, 25]. TLR4 signaling also requires MyD88 for inflammatory cytokine induction [17, 25]. Thus, the MyD88 pathway is a common and pivotal pathway for IL-1R and TLR signaling. Type I IFN induction and NK cell activation are not impaired in MyD88–/– mice when the mice are stimulated with the TLR3 ligand polyI:C (a dsRNA analog) [9]. However, in vitro, NK activation via mDC TLR2 stimulation is abolished in MyD88–/– cells [24], as discussed later. The physiological importance of MyD88 in NK activation in vivo in a variety of TLR pathways remains to be proven.

Signaling Pathways That Operate through the TICAM-1 (TRIF) Adaptor

TLR3 stimulation induces a unique output of IFN-β induction [31]. TLR3 binds the adaptor TICAM-1 [32, 33]. Overexpressed TICAM-1 induces IFN-β, suggesting the importance of this adaptor in selecting the IFN-β-inducing pathway (fig. 1). Ultimately, this adaptor also acts in the TLR4 IFN-β-inducing pathway [34, 35]. These results are consistent with those obtained from TRIF–/– mice [33].

IRF-3 activation induced by TLR4 signaling is independent of MyD88 and TIRAP/Mal, and instead depends on TICAM-1. To be more exact, another adaptor called TRAM (TICAM-2) serves as a bridging adaptor between TLR4 and TICAM-1 [34, 35]. TICAM-2 is similar to TICAM-1 in terms of the amino acid sequence of its Toll/IL-1 receptor homology (TIR) domain (fig. 1). In TICAM-2-deficient mice, both the MyD88-dependent pathway and the MyD88-independent pathway downstream of TLR4 signaling are defective, but TLR3 signaling remains intact [33]. TLR3 signaling also induces IRF-3 activation and IFN-β production through direct coupling with TICAM-1 [32, 34]. The IRF-3-activating pathway by TLR3/4 is greatly impaired in TICAM-1-deficient cells [36]. Thus, TICAM-1 is critically involved in TLR3 and TLR4 signaling.

Two noncanonical Iκ-B kinase homologs, i.e. Iκ-B kinase-epsilon (IKKε) (also known as inducible IKK) [36, 37] and TRAF family member-associated NF-κB activator (TANK)-binding kinase-I [TBK-I; also known as NF-κB-activating kinase (NAK)], are involved in TICAM-1-induced IRF-3 activation (fig. 1). These kinases physically couple with regulatory molecules, i.e. NAK-associated protein 1 (NAP1) or SINTBAD, and the complex can associate with TICAM-1 and induce phosphorylation and nuclear localization of IRF-3 [38, 39]. Furthermore, IRF-3-dependent gene expression induced by TLR3 and TLR4 signaling has been found to be defective in TRAF3–/– cells. Thus, TICAM-1 associates with TRAF3 for TLR3 and TLR4 signaling, causing the activation of IKKε/TBK-1 and IRF-3 [40, 41].

Signaling Pathways That Operate through the IPS-1 (MAVS, Cardif) Adaptor

Since most RNA viruses replicate in the cytoplasm, membrane proteins including TLR cannot recognize virus-replicating dsRNA in the cytoplasm. RIG-I, a member of the RNA helicase family of PRRs, has been identified with ubiquitous distribution in the cytoplasm [42]. RIG-I contains 2 caspase-recruiting domain (CARD)-like domains, i.e. DExD/H box RNA helicase and RHIM domains [42]. The helicase and RHIM domains interact with dsRNA or the 5′-triphosphate of virus-derived RNA with a short RNA duplex, whereas the CARD-like domains are required for interaction with IPS-1, an adaptor for activating downstream signaling pathways [17]. Furthermore, 2 additional RIG-I-like RNA helicases have been identified: MDA5 and LGP2 [43, 44]. MDA5 contains 2 CARD-like domains and a helicase domain, similar to RIG-I. MDA5 recognizes relatively long dsRNA in cytoplasm, whereas LGP2 lacks the CARD-like domains and is thought to positively regulate RIG-I and MDA5. These 2 signaling sensors recruit IPS-1, and IPS-1 induces the activation of IKKε/TBK-1 and IRF-3 [17] in a similar way to TICAM-1 (fig. 1).

IPS-1 contains a transmembrane domain that is rich in hydrophobic residues in its C-terminal tail and targets IPS-1 to the mitochondria [17, 45]. Notably, mitochondrial retention of IPS-1 is essential for IRF-3, IRF-7, and NF-κB activation, suggesting that signaling from mitochondria plays an important role in the antiviral immune response (fig. 1). The NS3/4A serine protease encoded by the hepatitis C virus has been demonstrated to target IPS-1 for cleavage [45, 46]. Using an in vitro cell culture infection system to introduce the hepatitis C virus, a putative cleavage site of IPS-1 was found to be located upstream of the transmembrane domain. The cleaved form of IPS-1, which lacks the transmembrane region, fails to activate IFN-β and nuclear factor (NF)-κB.

MDA5 detects long polyI:C or dsRNA, whereas RIG-I detects short dsRNA or the 5′-triphosphate end of RNA generated by viral polymerases [47, 48], indicating that these RNA helicases have different roles in the detection of viruses. Which of these pathways predominantly senses virus species is under examination. The simple interpretation that MDA5 is required for the recognition of picornaviruses and RIG-I recognizes that dsRNA-forming viruses [49] may be amended following the analysis of many virus species.

Mf, mDC, and fibroblast cells derived from RIG-I- or MDA5-deficient mice still displayed type I IFN induction in response to polyI:C stimulation, and the production of type I IFN was still observed in pDCs derived from IPS-1–/– mice [50, 51]. However, it is notable that the 2 pathways in the TLR system and the cytoplasmic IPS-1 pathway are required for dsRNA responses in different situations and cell types [50, 52]. Collectively, these observations indicate that the various modes of the RNA pattern-sensing system cooperate to detect cytoplasmic virus replication in a variety of tissues/organs.

NK Cell Activation via the TLR2/MyD88 Pathway in Mf and mDC

In vitro stimulation of mouse mDC or Mf with TLR2 stimulators BCG-CWS (cell wall skeleton) or Pam2 lipopeptide causes these cells to become NK activation inducers [24, 53]. Both TLR2 and MyD88 are indispensable for this type of NK cell activation. At least in in vitro studies, MyD88–/– Mf fail to reciprocally activate NK cells via cell-cell contact [54]. Mf MyD88 signaling through TLRs is reported to induce expression of the NKG2D ligand retinoic acid early induced transcript (RAE)-1 [55]. NK cells are then activated by MyD88-stimulated Mf. The NKG2D receptor on NK cells is downregulated by the RAE-1-NKG2D interaction [55]. MyD88 can be activated via the inflammasome pathway in human Mf [56] without the participation of TLR2. IL-1β or IL-18 (or possibly IL-33) liberated from *Plasmodium*-infected Mf may convert these cells into NK-activating Mf through IL-1β/IL-18 receptors [57, 58]. MyD88 in NK cells also participates in this type of NK activation [53, 59].

However, this is not the case in mDC. Although IL-12p70 is produced in mDC in response to the TLR2 ligand BCG-CWS, the role of IL-12 in NK activation is peripheral in the mouse system. There are at least 2 modes of MyD88-dependent NK cell activation in mDC: (1) MyD88 in pDC can couple with TLR7 or TLR9 to activate the IFN-α-inducing pathway, and this pathway also participates in NK cell activation, presumably through type I IFN liberated by pDC [60, 61]; (2) in contrast, MyD88–/– mDC lose the ability to mature in response to TLR2 ligands. For example, the TLR2 ligand Pam2CSK4 facilitates mDC-mediated NK cell activation in the case of wild-type mDC [24]. If wild-type mDC are replaced with MyD88–/– mDC, cell contact-mediated NK activation is hampered even when the cells are stimulated with Pam2 lipopeptide. Although TLR2–/– mDC largely abrogate the NK cell-activating function, slight functional activity remains in TLR2–/– mDC compared with MyD88–/– mDC, suggesting the presence of TLR2-independent MyD88-activating pathways, which may reflect the action of NOD-like receptor inflammatory pathways. Hence, NK cell activation proceeds through TLR2-dependent and TLR2-independent pathways of mDC, both of which involve MyD88. Furthermore, TLR2/MyD88 in NK cells minimally participates in direct NK activation [24, 53].

NK Cell Activation via the TICAM-1 or IPS-1 Pathways in mDC

It has long been established that a dsRNA analog, i.e. polyI:C, serves as an inducer of NK activation [62, 63]. In vivo administration of polyI:C to mice and in vitro exogenous addition of polyI:C to a mixture of BMDC and NK cells both resulted in activation of NK cells. Ex vivo studies using cells from KO mice have helped reveal how polyI:C activates NK cells. PolyI:C, unlike viral or in vitro-transcribed dsRNA, is internalized into the endosome and cytoplasm where it is recognized by TLR3 and MDA5, respectively [9, 11] (fig. 2). IPS-1 is the adaptor for MDA5/RIG-I, while TICAM-1 is the adaptor for TLR3 [32, 43, 64] (fig. 1). Using the gene-disrupted mouse cells of MDA5–/– and TLR3–/– or IPS-1–/– and TICAM-1–/– mDC, the pathway more involved in NK cell activation was examined in vitro [9–11]. In BMDC, the MDA5/IPS-1 pathway is more important than the TLR3/TICAM-1 pathway, as determined by the expression of NK activation marker CD69 and NK cytotoxicity [11], whereas the reverse is true in IFN-γ production by NK cells [64]. NK activation induced in mice injected with polyI:C is completely abrogated in double KO (IPS-1–/– and TICAM-1–/–) mice, suggesting that these 2 pathways are both required for polyI:C-mediated NK activation [10, 11].

Cell contact-mediated NK activation was found to dominantly occur in IRF-3 activation [64] and mDC by depletion studies [65]. Cytokines, including IL-12p40, IL-

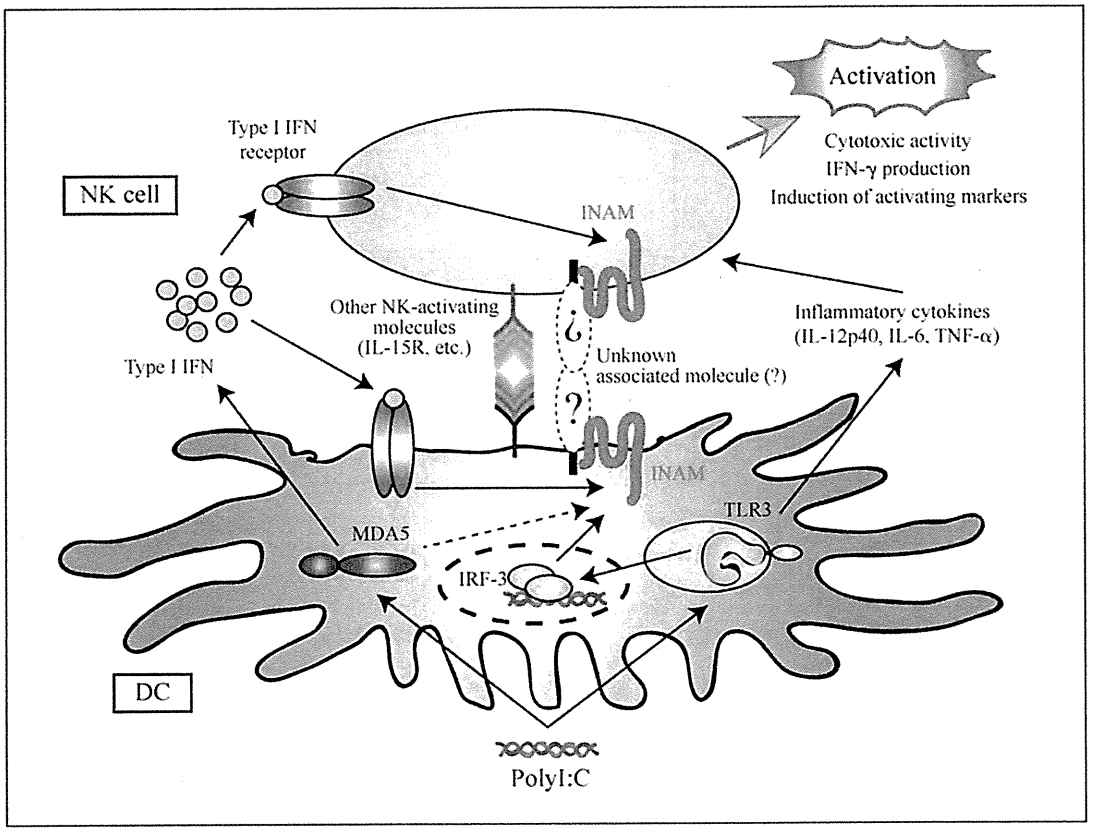

Fig. 2. Induction of INAM-mediated NK cell activation. PolyI:C-stimulated BMDC induce NK cell activation in vitro. INAM is responsible for BMDC contact-mediated NK activation. IRF-3 activation by polyI:C is crucial for INAM upregulation in BMDC, which results in the BMDC-mediated activation of NK cells. Upregulation of INAM on NK cells also facilitates NK cell activation in mDC-NK coculture.

15, and type I IFN, are produced in mDC in response to polyI:C stimulation. Both cell contact and cytokine production mediate NK activation in vitro but cytokines are known to be dispensable for NK activation by polyI:C-stimulated mDC in mice [64]. In vivo injected polyI:C allowed mice to mature splenic DCs [10, 11]. In vivo, bone marrow transplantation chimera analysis suggested that the TLR3-TICAM-1 pathway is important in myeloid cells, along with the IPS-1 pathway in nonmyeloid cells, in driving cytolytic activity by polyI:C [11] (fig. 3). Thus, nonmyeloid-derived soluble factors (mainly IFN-α/β) operate in NK activation in this case. In addition, splenic CD8α+ DC rather than CD8α– DC is crucial for driving NK activation via cell-cell contact [10].

The molecule responsible for mDC-NK contact activation has recently been investigated [64]. There are several polyI:C-inducible membrane-associated molecules in mDC and one of these molecules, designated INAM (IRF-3-dependent NK cell activating molecule), participates in mDC-NK reciprocal activation (fig. 2). However, when overexpressed in non-NK target cells, INAM does not act as an NK-activating ligand; it works only on mDC/Mf for NK activation. Since INAM is predicted to have a tetraspanin-like sequence, unidentified molecules coupling to INAM may foster mDC-NK contact.

NK Cell Activation in Humans and Mice

In human monocyte-derived DC [66] and mouse CD8α+-like human DC (BDCA3+/XCR1+) [67, 68], the early response to dsRNA (including polyI:C and polyA:U) induces the production of IL-12p40 and type I IFN via the TLR3/TICAM-1 pathway. These early-phase cytokines

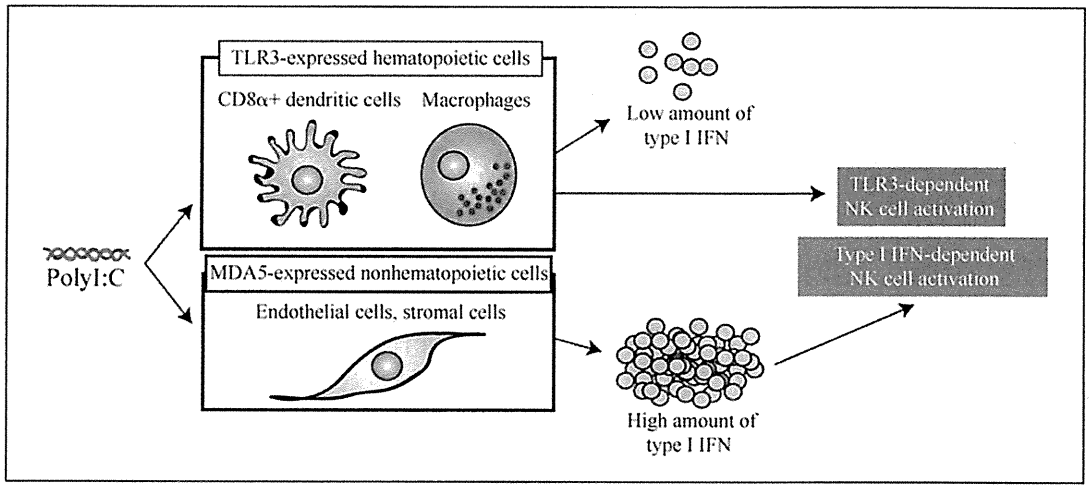

Fig. 3. The IPS-1 pathway in nonmyeloid cells and TLR3 in myeloid cells participate in the activation of NK cells. Mice i.v. injected with polyI:C activate NK cells. Two distinct pathways are involved in NK cell activation in this case: (1) TLR3 in Mf and mDC recognize the i.v. injected polyI:C and drive the internal NK activation pathway, and (2) nonhematopoietic cells recognize the polyI:C by their MDA5 in the cytoplasm to produce a large amount of type I IFN, which in turn activates Mf/DC for NK activation.

play a key role in priming NK cells to induce a low amount of IFN-γ in vitro. In vitro administered dsRNA also activates NK cells via direct stimulation of the RIG-I pathway in NK cells. In the following phase, mDC are recruited to draining lymph nodes to encounter NK cells. Subsequently, mDC-NK contact occurs inducing full maturation of NK cells. At this stage, NK cells engage in significant IFN-γ production. This recent observation is in close agreement with the results reported in human in vitro cocultured liver DC and NK cells [69]. These findings clearly reveal the importance of mDC IL-12p70 and the NK cell RIG-I pathway in NK cell priming in humans.

In mouse in vitro studies, mouse BMDC or CD8α+ splenic DC activate NK cells via cell-cell contact rather than IL-12p70 or type I IFN. BMDC, as well as CD8α+ splenic DC, express TLR3 mRNA, and polyI:C stimulation induces activation of both the TICAM-1 pathway and the IPS-1 pathway [9–11, 64]. IL-12p40 (which is likely the p40 dimer that inhibits IL-12R-derived signaling), instead of IL-12p70, is produced in response to polyI:C in mouse DC-NK coculture studies [64]. A membrane molecule, i.e. INAM, expressed secondary to IRF-3 activation in BMDC or CD8α+ DC stimulates initial DC-mediated NK activation. Full activation of NK cells, including cytolytic activity against target molecules, is provoked only secondarily to DC-NK contact in mice.

In contrast, in vivo studies on NK cell activation have been performed in mice via intravenous (i.v.) injection of polyI:C. NK activation occurs in response to i.v. injected polyI:C, and NK activation has been shown to depend on the MDA5/IPS-1 pathway and TLR3/TICAM-1pathway in KO mice [11] (fig. 3). Surprisingly, in bone marrow chimera studies, initial induction of type I IFN by MDA5 from nonmyeloid cells played a crucial role in splenic DC maturation. CD8α+ DC maturation secondarily triggered NK cell activation. If this is the case, myeloid cell TLR3 and nonmyeloid cell MDA5 actually participate in polyI:C-dependent maturation of splenic DC to drive NK activation, at least at an early phase of i.v. administration in vivo. Presumably, stromal or vascular endothelial cells are a source of MDA5-mediated type I IFN induction, which in turn activates splenic DC. There are several subsets of DC in the mouse spleen. Only CD8α+ DC express high TLR3 and participate in NK activation [10, 70]. On the other hand, intraperitoneal administration of polyI:C first activates Mf in the peritoneal cavity of mice [71]. The route of polyI:C administration may therefore stimulate different RNA sensors to activate IRF-3. IL-12p70 has a minimal role in mouse DC-mediated NK activation.

It is currently unclear which mode of NK activation, TICAM-1 or IPS-1, is dominant in other mammals and vertebrates. NK cells and the TICAM-1 and IPS-1 pathways are conserved across vertebrates. Differential re-

sponses to polyI:C in cytokine production and NK activation in other animals may be partly due to the systemic differences in RNA recognition in different cell types and tissues.

Perspectives

NK cell activation is an important event in the immune response to cancer or infectious diseases. Recent molecular/cellular analyses suggest that the cells and molecular mechanisms involved in NK cell activation differ between in vivo PAMP-stimulated mice and in vitro PAMP-stimulated cell cocultures. A discrepancy also appears in in vitro NK activation studies in humans and mice. The basal IFN-inducing properties have been reported in mice but not in humans [6]. The response to PAMP also differs depending on the delivery routes. Caution is therefore necessary when adapting the results from KO mice to human clinical studies. Only 5 μg of polyI:C sufficiently induces IFN-β from human fibroblasts which express TLR3 on the cell surface, but >150 μg is still insufficient for mouse fibroblasts [72, 73]. Although polyI:C is effective for NK activation, it often induces a life-threatening cytokine storm in mice [74]. A future aim is to activate NK cells with no side effects in human patients. If this can be achieved, NK cell activation could be applied to the establishment of effective vaccines and immunotherapies.

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References

1 Trinchieri G: Biology of natural killer cells. Adv Immunol 1989;47:187–376.
2 Bryceson YT, March ME, Ljunggren HG, Long EO: Activation, coactivation, and costimulation of resting human natural killer cells. Immunol Rev 2006;214:73–91.
3 Moretta A: Natural killer cells and dendritic cells: rendezvous in abused tissue. Nat Rev Immunol 2002;2:957–964.
4 Granucci F, Zanoni I, Ricciardi-Castagnoli P: Central role of dendritic cells in the regulation and deregulation of immune responses. Cell Mol Life Sci 2008;65:1683–1697.
5 Joffre O, Nolte MA, Spörri R, Reis e Sousa C: Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunol Rev 2009;227:234–247.
6 Takaoka A, Taniguchi T: New aspects of IFN-α/β signalling in immunity, oncogenesis and bone metabolism. Cancer Sci 2003; 94:405–411.
7 Kono H, Rock KL: How dying cells alert the immune system to danger. Nat Rev Immunol 2008;8:279–289.
8 Seya T, Shime H, Ebihara T, Oshiumi H, Matsumoto M: Pattern recognition receptors of innate immunity and their application to tumor immunotherapy. Cancer Sci 2010;101:313–320.
9 Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, Tsujimura K, Takahashi T, Ikawa M, Okabe M, Inoue N, Okamoto-Tanaka M, Ishizaki H, Miyoshi J, Matsumoto M, Seya T: Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci USA 2007;104:252–257.
10 Miyake T, Kumagai Y, Kato H, Guo Z, Matsushita K, Satoh T, Kawagoe T, Kumar H, Jang MH, Kawai T, Tani T, Takeuchi O, Akira S: Poly I:C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways. J Immunol 2009;183:2522–2528.
11 McCartney S, Vermi W, Gilfillan S, Cella M, Murphy TL, Schreiber RD, Murphy KM, Colonna M: Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J Exp Med 2009;206:2967–2976.
12 Lanier LL: Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008;9:495–502.
13 Dimasi N, Moretta L, Biassoni R: Structure of the Ly49 family of natural killer (NK) cell receptors and their interaction with MHC class I molecules. Immunol Res 2004;30:95–104.
14 Sun JC, Beilke JN, Lanier LL: Adaptive immune features of natural killer cells. Nature 2009;457:557–561.
15 Cooper MA, Colonna M, Yokoyama WM: Hidden talents of natural killers: NK cells in innate and adaptive immunity. EMBO Rep 2009;10:1103–1110.
16 Newman KC, Riley EM: Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat Rev Immunol 2007;7:279–291.
17 Kawai T, Akira S: The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 2009;21:317–337.
18 Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL: Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science 2002;296:1323–1326.
19 Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, Strominger JL, Yewdell JW, Porgador A: Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human natural killer cells. Nature 2001;409:1055–1060.
20 Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, Wagner H, Kirschning CJ, ter Meulen V, Schneider-Schaulies S: Hemagglutinin protein of wild-Type measles virus activates Toll-like receptor 2 signaling. J Virol 2002;76:8729–8736.

21 Uehori J, Fukase K, Akazawa T, Uematsu T, Akira S, Funami S, Shingai M, Matsumoto M, Azuma I, Toyoshima K, Kusumoto S, Seya T: Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation. J Immunol 2005;174:7096–7103.
22 Korbel DS, Newman KC, Almeida CR, Davis DM, Riley EM: Heterogenous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. J Immunol 2005;175:7466–7473.
23 Becker I, Salaiza N, Aguirre M, Delgado J, Carrillo-Carrasco N, Kobeh LG, Ruiz A, Cervantes R, Torres AP, Cabrera N, González A, Maldonado C, Isibasi A: Leishmania lipophosphoglycan (LPG) activates NK cells through Toll-like receptor-2. Mol Biochem Parasitol 2003;130:65–74.
24 Azuma M, Sawahata R, Akao Y, Ebihara T, Yamazaki S, Matsumoto M, Hashimoto M, Fukase K, Fujimoto Y, Seya T: The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation. PLoS One 2010;5:e12550.
25 Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S: Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 1998;9:143–150.
26 Kawai T, Adachi O, Ogawa T, Takeda K, Akira S: Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999;11:115–122.
27 Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, Taya C, Taniguchi T: Spatiotemporal regulation of MyD88-IRF-7 signaling for robust type I interferon induction. Nature 2005;434:1035–1040.
28 Uematsu S, Akira S: Toll-like receptors and type I interferons. J Biol Chem 2007;282:15319–1532.
29 Honda K, Taniguchi T: IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 2006;6:644–658.
30 Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano S, Honda K, Ohba Y, Mak TW, Taniguchi T: Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 2005;434:243–249.
31 Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T: Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 2003;171:3154–3162.
32 Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T: TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 2003;4:161–167.
33 Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S: Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;301:640–643.
34 Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T: TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 2003;278:49751–49762.
35 Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT: LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the Toll adapters TRAM and TRIF. J Exp Med 2003;198:1043–1055.
36 Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T: IKK-epsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491–496.
37 Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J: Triggering the interferon antiviral response through an IKK-related pathway. Science 2003;300:1148–1151.
38 Sasai M, Oshiumi H, Matsumoto M, Inoue N, Fujita F, Nakanishi M, Seya T: Cutting edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. J Immunol 2005;174:27–30.
39 Ryzhakov G, Randow F: SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. EMBO J 2007;26:3180–3190.
40 Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Häcker G, Mann M, Karin M: Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 2006;439:204–207.
41 Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, Perry A, Cheng G: Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature 2006;439:208–211.
42 Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T: The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730–737.
43 Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A, Fujita T: Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 2005;175:2851–2858.
44 Saito T, Hirai R, Loo YM, Owen D, Johnson CL, Sinha SC, Akira S, Fujita T, Gale M Jr: Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci USA 2007;104:582–587.
45 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 USA 2005;102:17717–17722.
46 Loo YM, Owen DM, Li K, Erickson AK, Johnson CL, Fish PM, Carney DS, Wang T, Ishida H, Yoneyama M, Fujita T, Saito T, Lee WM, Hagedorn CH, Lau DT, Weinman SA, Lemon SM, Gale MJ: Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci USA 2006;103:6001–6006.
47 Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G: 5′-Triphosphate RNA is the ligand for RIG-I. Science 2006;314:994–997.
48 Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C: RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 2006;314:997–1001.
49 Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S: Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441:101–105.
50 Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S: Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005;23:19–28.
51 Kochs G, Bauer S, Vogt C, Frenz T, Tschopp J, Kalinke U, Waibler Z: Thogoto virus infection induces sustained type I interferon responses that depend on RIG-I-like helicase signaling of conventional dendritic cells. J Virol 2010;84:12344–12350.
52 Matsumoto M, Oshiumi H, Seya T: Antiviral responses induced by the TLR3 pathway. Rev Med Virol 2011, Epub ahead of print.
53 Martinez J, Huang X, Yang Y: Direct TLR2 signaling is critical for NK cell activation and function in response to vaccinia viral infection. PLoS Pathog 2010;6:e1000811.
54 Sawaki J, Tsutsui H, Hayashi N, Yasuda K, Akira S, Tanizawa T, Nakanishi K: Type 1 cytokine/chemokine production by mouse NK cells following activation of their TLR/MyD88-mediated pathways. Int Immunol 2007;19:311–320.
55 Hamerman JA, Ogasawara K, Lanier LL: Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. J Immunol 2004;17:2001–2005.

56 Cole LE, Santiago A, Barry E, Kang TJ, Shirey KA, Roberts ZJ, Elkins KL, Cross AS, Vogel SN: Macrophage proinflammatory response to *Francisella tularensis* live vaccine strain requires coordination of multiple signaling pathways. J Immunol 2008;180:6885–6891.
57 Adachi K, Tsutsui H, Kashiwamura S, Seki E, Nakano H, Takeuchi O, Takeda K, Okumura K, Van Kaer L, Okamura H, Akira S, Nakanishi K: *Plasmodium berghei* infection in mice induces liver injury by an IL-12- and Toll-like receptor/myeloid differentiation factor 88-dependent mechanism. J Immunol 2001;167:5928–5934.
58 Baratin M, Roetynck S, Lépolard C, Falk C, Sawadogo S, Uematsu S, Akira S, Ryffel B, Tiraby JG, Alexopoulou L, Kirschning CJ, Gysin J, Vivier E, Ugolini S: Natural killer cell and macrophage cooperation in MyD88-dependent innate responses to *Plasmodium falciparum*. Proc Natl Acad Sci USA 2005; 102:14747–14752.
59 Akao Y, Ebihara T, Masuda H, Saeki Y, Akazawa T, Hazeki K, Hazeki O, Matsumoto M, Seya T: Enhancement of antitumor natural killer cell activation by orally administered Spirulina extract in mice. Cancer Sci 2009; 100:1494–501.
60 Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, Orihuela MM, Akira S, Yokoyama WM, Colonna M: TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity 2004;21:107–119.
61 Hoshino K, Sugiyama T, Matsumoto M, Tanaka T, Saito M, Hemmi H, Ohara O, Akira S, Kaisho T: IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. Nature 2006; 440:949–953.
62 Djeu JY, Heinbaugh JA, Holden HT, Herberman RB: Augmentation of mouse natural killer cell activity by interferon and interferon inducers. J Immunol 1979;122: 175–181.
63 Wiltrout RH, Salup RR, Twilley TA, Talmadge JE: Immunomodulation of natural killer activity by polyribonucleotides. J Biol Response Mod 1985;4:512–517.
64 Ebihara T, Azuma M, Oshiumi H, Kasamatsu J, Iwabuchi K, Matsumoto K, Saito T, Taniguchi T, Matsumoto M, Seya T: Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. J Exp Med 2010;207:2675–2687.
65 Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A: Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity 2007;26:503–517.
66 Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T: Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 2003;171:3154–3162.
67 Crozat K, Guiton R, Contreras V, Feuillet V, Dutertre CA, Ventre E, Vu Manh TP, Baranek T, Storset AK, Marvel J, Boudinot P, Hosmalin A, Schwartz-Cornil I, Dalod M: The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J Exp Med 2010;207:1283–1292.
68 Bachem A, Güttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, Salama A, Movassaghi K, Opitz C, Mages HW, Henn V, Kloetzel PM, Gurka S, Kroczek RA: Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 2010;207:1273–1281.
69 Tu Z, Bozorgzadeh A, Pierce RH, Kurtis J, Crispe IN, Orloff MS: TLR-dependent cross talk between human Kupffer cells and NK cells. J Exp Med 2008;205:233–244.
70 Jelinek I, Leonard JN, Price GE, Brown KN, Meyer-Manlapat A, Goldsmith PK, Wang Y, Venzon D, Epstein SL, Segal DM: TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. J Immunol 2011;186:2422–2429.
71 Wu CY, Monie A, Yang HY, Wu Tc, Hung CF: Intraperitoneal administration of polyI:C with polyethylenimine leads to significant antitumor immunity against murine ovarian tumors. Cancer Immunol Immunother 2011, in press.
72 Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T: Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Commun 2002;293:1364–1369.
73 Matsumoto M, Seya T: TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 2008;60:805–812.
74 Absher M, Stinebring WR: Toxic properties of a synthetic double-stranded RNA: endotoxin-like properties of poly I – poly C, an interferon stimulator. Nature 1969;223:715–717.

Meeting Summary

Will There Be an HCV Meeting in 2020? Summary of the 17th International Meeting on Hepatitis C Virus and Related Viruses

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Hepatitis C virus (HCV), which was discovered in 1989, is a major etiologic agent in human liver disease. Approximately 130 million people, or 2% of the population, worldwide are infected. The 17th International Meeting on Hepatitis C Virus and Related Viruses was held September 10–14, 2010, in Yokohama, Japan. The meeting was attended by almost 700 scientists from all over the world who are interested in the fundamental aspects of the molecular virology, immunology, pathogenesis, prevention, and treatment of HCV infection. Two special opening lectures given by Masaaki Komatsu and Takashi Gojobori focused attention on the related research fields of autophagy and genome biology, respectively. In the subsequent sessions, the latest research, original studies, and controversies were presented in 9 keynote lectures, 82 oral presentations, and 329 poster presentations.

Viral Entry

The opening scientific session of this meeting focused on the viral host cell entry processes. Thomas Baumert presented the keynote lecture, which included an overview of the HCV cell entry process and recent advances at his laboratory. These included the finding that HCV variants that reinfect the liver after transplantation demonstrate more efficient cell entry and are less susceptible to neutralization by host antibodies. He also described the isolation of monoclonal antibodies against claudin-1 that do not inhibit either extracellular or direct cell-to-cell HCV transfer.

Alexander Ploss described the establishment of a mouse model for studying HCV cell entry. They utilized an HCV cell culture virus (HCVcc) expressing recombinase and transgenic mice bearing a recombinase-activatable fluorescent protein. Bioluminescent imaging indicated that only mice transduced with CD81 and occludin supported HCVcc entry. The presence of an intact immune system in these animals makes it particularly important for the testing of HCV vaccine candidates. Danyelle N. Martin described a role for transferrin receptor 1 (TfR1) in mediating HCV cell entry. The inhibition of HCV entry with TfR1 antibodies and silencing, suggest this factor should be added to the growing list of cellular proteins required for HCV cell entry. Joachim Lupberger presented results from a study showing an essential role for the epidermal growth factor receptor (EGFR) in HCV cell entry. He found that EGFR is required for both mediating the interactions between two other entry factors, CD81 and CLDN1, and catalyzing the fusion activity of viral glycoproteins.

Translation/Replication

Volker Lohmann began the session by describing what is known of the functions of viral nonstructural proteins and their associated host cellular factors in viral translation and replication. He included an overview of viral isolates and model systems currently used, and presented data addressing the mechanisms for efficient replication of the JFH-1 isolate.

Several reports have focused on the molecular basis of the architecture and composition of membrane-associated sites for HCV replication, which often induce membrane alterations, such as the so-called membranous web. Brenno Wolk demonstrated that NS4B is sufficient to direct all nonstructural proteins into the viral replication complex compartment, and that intragenotype-specific interactions are required for NS4B-dependent recruitment of NS5A. Ines Romero-Brey showed that the membranous web predominantly contains double-membrane vesicles with various diameters. These vesicle structures were connected to the endoplasmic reticulum (ER) through funnel-like structures.

Several DDX DEAD-box RNA helicases were identified as host factors associated with HCV replication. Yasuo Ariumi presented the cross-talk of HCV with DDX proteins and the role of distinct DDX proteins in viral replication. Tetsuro Shimakami and Selena M. Sagan reported the importance of miR-122 to not only enhance IRES-mediated translation, but stabilize positive-strand HCV RNA by binding to its 5′ extremity. Enzymatic activity of host phosphatidyl-inositol-4 kinase III alpha was shown to be critically involved in HCV replication and the activity is regulated by HCV NS5A (Simon Reiss). Nam-Joon Cho reconstituted a functionally active full-

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length HCV polymerase on a biomimetic membrane platform. Deborah Harrus found that guanosine triphosphate specifically stimulates the initial step of de novo initiation by stimulating transition of newly formed linker primer.

Assembly and Release

In the keynote lecture, Guangxiang G. Luo presented an overview of particle assembly and release, and the impact of apolipoprotein (Apo) E in the entry and assembly of HCV. He demonstrated the inhibition of HCVcc entry by treatment with anti-ApoE antibody and the direct interaction of ApoE with NS5A.

Ann L. Wozniak showed an important role for p7 in the production of infectious particles. Their data suggest that p7 stimulates virus production through the alkalization of intracellular vesicles. Ophelia Granio showed that both p7 and NS2 are required for the recruitment of core from lipid droplets (LDs) to ER. Costin-Ioan I. Popescu showed that NS2 accumulated in dotted structures in the ER in juxtaposition with Core and LDs. They concluded that cross-talk among Core, E1, E2, p7, and NS2 was essential for virion assembly. Vlastimil Jirasko demonstrated point mutations in the transmembrane regions of NS2 impaired the particle production and suggested that NS2 serves as a platform of viral and cellular proteins that coordinates HCV assembly. Qisheng Li identified the proviral function of IKK by genome wide siRNA screening. IKK regulates lipid metabolism and biogenesis of LDs and may enhance production of virus particles. The very low-density lipoproteins are secreted via a Golgi-dependent pathway. Bryan R. Bishe demonstrated the important role of phosphatidylinositol-4-phosphate and its interacting protein GOLPH3 in HCV secretion in the trans-Golgi network. Roland Remenyi showed 3-dimensional visualization of the HCV life cycle in cultured cells by electron tomography. They detected virus-like particles at various cytoplasmic locations. Viral particles in the proximity of LDs and within sponge-like inclusion were observed. These results provide ultrastructural visualization of putative assembly sites close to LDs.

Host Factors

In the invited lecture, Sara Cherry presented an overview of high-throughput screening toward the identification of host factors required for viral infection.

The contribution of autophagy to the HCV life cycle was also presented in this section, most notably, host factors linked with lipids. Tsubasa Munakata showed that the fatty acid synthase is required for efficient HCV replication. They also suggested the importance of palmitate for HCV replication. Samantha L. Blackham presented both the thioredoxin-interacting protein and the peroxisome proliferator activated receptor- have significant effects on HCV replication. The host factors functioning on infectious HCV particle production were also reported. Takayuki Hishiki demonstrated the isoform dependent binding affinities of ApoE for low-density lipoprotein receptors and they affect infectivity of HCV. Laurent Chatel-Chaix found that Y-box binding protein interacted with HCV NS3 protein and viral RNA and was relocalized from nucleocytoplasmics site to the core-containing surface of LDs. Mohsan Saeed reported that the ER-associated degradation pathway was activated by HCV infection in a viral envelope protein-dependent manner. Po-Yuan Ke showed that HCV infection induces the unfolded protein response and activates the autophagic pathway. They proposed that autophagy contributes to the suppression of HCV in an autolysosome formation-dependent manner. Hiroto Kambara did not find any effects on HCV replication by inhibition of autophagosome formation in replicon cells. They proposed a role for autophagy induced by HCV infection to avoid the generation of vacuolation harmful to cell survival. Qisheng Li reported the network map of cellular pathways and machineries that are associated with HCV life cycle.

Very low-density lipoprotein is now considered to be one of a component of HCV particles. LDs are composed of fatty acid, triglyceride, and cholesterol, surrounded by several types of lipoproteins. In addition, Daniel J. Felmlee reported that chylomicron-associated viruses may be generated by virion association while in the vascular compartment. Francois Jean showed that the serine protease inhibitor protein Spn4A was modified to be directed to Site-1 protease specifically and was introduced into adenovirus vector to inhibit cholesterol and fatty acid syntheses for down-regulation of HCV propagation. The modified serpin could suppress Site-1 protease activity, reduce the LD, and block HCVcc infection. Nicolas Menzel tried to identify novel cellular factors involved in HCV assembly and release and found ERK inhibitor and cytosolic phospholipase A2 (cPLA2) inhibitor reduce viral production. cPLA2 inhibitor also reduced the amount of LD-associated core and supernatant ApoB/E. cPLA2 may be crucial for assembly of infectious HCV particles, possibly through participating in the formation of lipoproteins. Kohji Moriishi reported that the proteasome activator PA28 participates in HCV propagation. PA28 may participate in the propagation of HCV by regulating the degradation of Core in both ubiquitin-dependent and -independent manners. NS5A is regulated by phosphorylation of several host protein kinases. Takahiro Masaki identified 79 serine threonine protein kinases that were tightly associated with NS5A. Two of these may regulate the production of viral particles and/or viral replication.

Innate Immunity

The early phase of host defense against viral infection has largely been delineated based on recent advances in innate immunity. In the invited lecture, Manoj N. Krishnan introduced his comprehensive study on the Toll-like receptor 3-TRIF (TICAM-1) pathway. Using RNAi and polyI:C, he screened the genes specifically upregulated via the TRIF (TICAM-1) pathway. He expected that some viral infections are selectively blocked by the IPS-1 pathway, while others are blocked by the TRIF pathway.

Michael Gale, Jr., identified IFITM1 inhibits HCV infection. IFITM1 assembles with CD81 and tranlocates to the tight junction. This translocation of CD81 hampers the receptor function of CD81. They also discovered a novel pathway for ISGF3 activation. A non-receptor type tyrosine kinase-1 triggers activation of ISGF3 independent from the classical IFNAR pathway. IP-10 is a chemokine and is a negative predictor for pegylated interferon (IFN)/ribavirin therapy. Matthew L. Albert indicated that there is a 2-amino-acid-deleted form of IP-10 that serves as an antagonist for intact IP-10, and this form abrogates an early virologic response. As this IP-10 truncation is mediated by dipeptidylpeptidase IV, they believed that dipeptidylpeptidase IV is a novel therapeutic target for HCV patients during IFN therapy. Joo Chun Yoon suggested that activation of natural killer cells is inhibited by HCV-infected hepatocytes. They claimed that the early phases of HCV infection may be established through the failure of virus-inducible natural killer cell activation. Shin-ichiro Nakagawa reported that polyI:C induces both type I IFN and IFN- in human hepatocytes. The antiviral effect appears to parallel the induction of IFN- . This, together with the report by Emmanuel Thomas, suggests that the IFN- system is activated in HCV infected hepatocytes.

Adaptive Immunity

In a keynote lecture, Robert Thimme summarized the mechanisms of HCV-induced T-cell dysfunction. Multifaceted factors contribute to the hyporesponsiveness of T cells, including viral mutations, primary T-cell failure, lack of support from dendritic cells, expression of inhibitory molecules on T cells, and abundance of regulatory T cells (Tregs). Whether the ability of HCV-specific CTLs is comparable with that of CTLs having other specificities remains controversial. Bianca Seigel showed that HCV-specific CTLs are functionally impaired when compared with other CTLs, irrespective of their expression of inhibitory receptors or differentiation stages. CD161 is a C-type lectin that is expressed in HCV-specific CD8 T cells with tissue homing phenotype. Vicki M. Fleming found that CD4 CD161 T cells produce large amounts of inflammatory cytokines and accumulate in the liver, where they are thought to exert pro-inflammatory roles. Naruyasu Kakita reported that certain adaptive Tregs, known as interleukin (IL)-10-producing type 1 Tregs, are increased in HCV-positive hepatocellular carcinoma patients, and their significance in hepatocellular carcinoma was greater than that of natural Tregs. Even in patients who have attained a sustained virologic response, trace amounts of HCV RNA are sporadically detectable in plasma. Barbara Rehermann reported the inoculation studies of such plasma. Residual HCV RNA in patients was able to infect chimpanzees and induced broad, HCV-specific T-cell responses. HCV RNA levels continued to be high when T-cell responses declined, suggesting that such HCV remains transmissible as hepatotropic pathogens.

Pathogenesis

In the invited lecture, Michael Diamond presented new mechanisms for West Nile virus immune evasion via 2'O methylation of viral RNA to subvert host innate immunity.

Genome-wide analysis of quantitative data (transcriptomics, proteomics, and metabolomics) facilitates systems biology analysis of HCV infection. Deborah L. Diamond analyzed the pathways involved in the progression of chronic hepatitis, namely, fibrosis and carcinogenesis, and found that molecules relating to cell metabolism including fatty acid oxidation enzymes and antioxidant systems may be master regulators of liver disease progression in HCV infection. HCV core protein has been shown to play a key role in the development of steatosis in HCV infected liver, especially in patients with genotype 3a HCV infection. Sophie Clement-Leboube showed that PTEN expression was down-regulated in the HCV infected liver. Analysis of lipo-viral-particle from hepatitis C patients by Olivier Diaz revealed that empty lipo-viral-particle lacking HCV RNA outnumbers those with RNA. The presence of virus-modified lipoproteins in HCV-infected patients may play a role in the pathogenesis of hepatitis C. Massimiliano Pagani used serum miRNA signatures to monitor liver disease in HCV infection and found miRNome candidates that are specific for HCV disease progression. Shuhei Taguwa showed that Con1 replicon induces incomplete autophagy through the dysfunction of autolysosomal acidification, which results in the secretion of immature cathepsin B in cells. Because the secretion of the protein is enhanced in many types of tumors, this observation may be associated with the pathogenesis of liver tumorigenesis in HCV infection.

The existence of extrahepatic manifestations is another issue of interest. Essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis, and Sjögren syndrome are conditions that have been shown to correlate

with HCV infection. Nicola A. Fletcher reported that brain microvascular endothelial cells express all the recognized entry factors for HCV, and brain microvascular endothelial cells actually support infection by HCVpp and HCVcc. This suggests potential disorders of the central nervous system in HCV infection.

Treatment

In the keynote lecture, Masashi Mizokami presented "Genome-wide association study and its application for HCV treatment." He emphasized that the functional relevance of IL-28B single nucleotide polymorphisms should be elucidated to further advance the progress of research on the mechanisms of chronic HCV infection and treatment.

Yasuhiro Asahina presented that genetic variation in IL-28B is associated with gene expression involving innate immunity. Minor alleles of IL-28B, as well as a higher RIG-I/IPS-1 ratio are associated with null viral response. Martin Laggins correlated IL-28B genetic variation with pretreatment levels of IP-10 and HCV RNA throughout therapy. The favorable genetic variation of IL-28B single nucleotide polymorphisms (major allele) was significantly associated with lower baseline IP-10. Masao Honda revealed that hepatic IFN-stimulated genes (ISGs) are associated with genetic variation in IL-28B and the outcome of IFN therapy for chronic hepatitis C using microarray gene expression profiling of the biopsied liver samples. Multivariate logistic regression analysis showed that ISGs, fibrosis stage, and ISDR mutations were strongly associated with viral response. Hepatic ISGs were associated with the IL-28B polymorphism and expression was significantly higher in patients with the minor genotype than in those with the major genotype. Takashi Motomura also analyzed ISG expression using liver transplantation samples. Expression of ISGs in recipients' liver carrying the minor allele of IL-28B was significantly up-regulated when compared with the major allele. Surprisingly, IFN sensitivity for recurrent hepatitis C after liver transplantation is influenced by IL-28B genetic variation not only in recipients, but also in donors.

Drug Development

This session opened with a keynote lecture by Raffaele De Francesco describing the current state of drug development for patients with chronic hepatitis C. Because of the rapid development of NS3/4A, NS5A, and NS5B inhibitors, he finally presented the hopeful message "Will there be an HCV meeting in 2020?".

Lotte Coelmont characterized an NS5A D320E variant showing low-level resistance to DEB025, a cyclophilin (Cyp)-binding molecule. This study suggests that DEB025 presents a high barrier to resistance, and that D320E confers low-level resistance to DEB025 by reducing the need for CypA-dependent isomerization of NS5A. Paul Targett-Adams reported that NS5A inhibitors stimulated redistribution of NS5A from the ER to ring-like structures in the cytoplasm, and disrupted colocalization with NS5B. This study suggests that NS5A inhibitors perturb formation of new replication complexes rather than acting on preformed complexes. Luis M. Schang developed a family of small synthetic rigid amphiphiles with large hydrophilic heads and small, planar and rigid hydrophobic tails, called RAFIs (rigid amphipathic fusion inhibitors), which inhibit the infectivity of enveloped virions including HCV. Emmanuel Thomas screened host genes involving the anti-HCV activity of ribavirin. Among 64 host genes, several candidate genes were identified as host factors involving ribavirin's anti-HCV activity. Interestingly, silencing of the *ITPA* gene increased the anti-HCV activity of ribavirin. Pablo Gastaminza identified a novel family of 1,2-diamines as an anti-HCV reagent from a chemical library. The analysis of 300 derivatives identified several compounds with enhanced potency and low cytotoxicity.

Vaccines/Epidemiology

HCV therapeutic vaccines are aimed to induce effective T-cell responses. Marianne Mikkelsen reported that vaccination of mice with recombinant adenovirus expressing HCV NS3 fused to the MHC class II chaperon protein invariant chain significantly enhanced NS3 specific CD8 T-cell responses, and protected mice against NS3-expressing vaccinia virus challenge. This vaccination induced polyfunctional CD8 memory T cells. Lars Frelin aimed to restore immunologic function through vaccination in a transgenic mouse model with impaired HCV-specific T-cell responses owing to a persistent presence of hepatic HCV NS3/4A antigens. They found that heterologous sequences improved activation and expansion of NS3/4A-specific T cells in a wild-type host, as well as in a tolerant NS3/4A-transgenic mouse model. The authors also suggested an important role for Tregs in the impaired HCV-specific T-cell responses.

Livia M.G. Rossi examined antibody cross-immunoreactivity against different HVR1 variants to identify antigens with a possible application of HCV vaccine development. The authors identified a small set of HVR1 variants that cross-immunoreacted with a large number of HVR1 peptides, thus suggesting their potential use in the development of HCV vaccine candidates.

Conclusion

HCV2010 in Yokohama was successful and contributed to the progress of research in the field. HCV infection remains one of the most serious worldwide health problems. The goals of this symposium were to

increase the scientific understanding of this virus and gain insights applicable to future efforts to control its infection. From this point of view, we gained further fundamental understanding about HCV at the meeting. The discovery of IL-28B as a new host factor involved in HCV treatment and pathogenesis had a major impact on HCV research. New treatment advances have been made in recent years and will continue in the near future. We would like to conclude that this meeting was successful in providing opportunities for exchanging up-to-date information and international collaboration. The next meeting will take place in Seattle, Washington, from September 8–12, 2011 (http://www.hcv2011.org/).

Reprint requests

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Conflicts of interest

The authors disclose no conflicts.