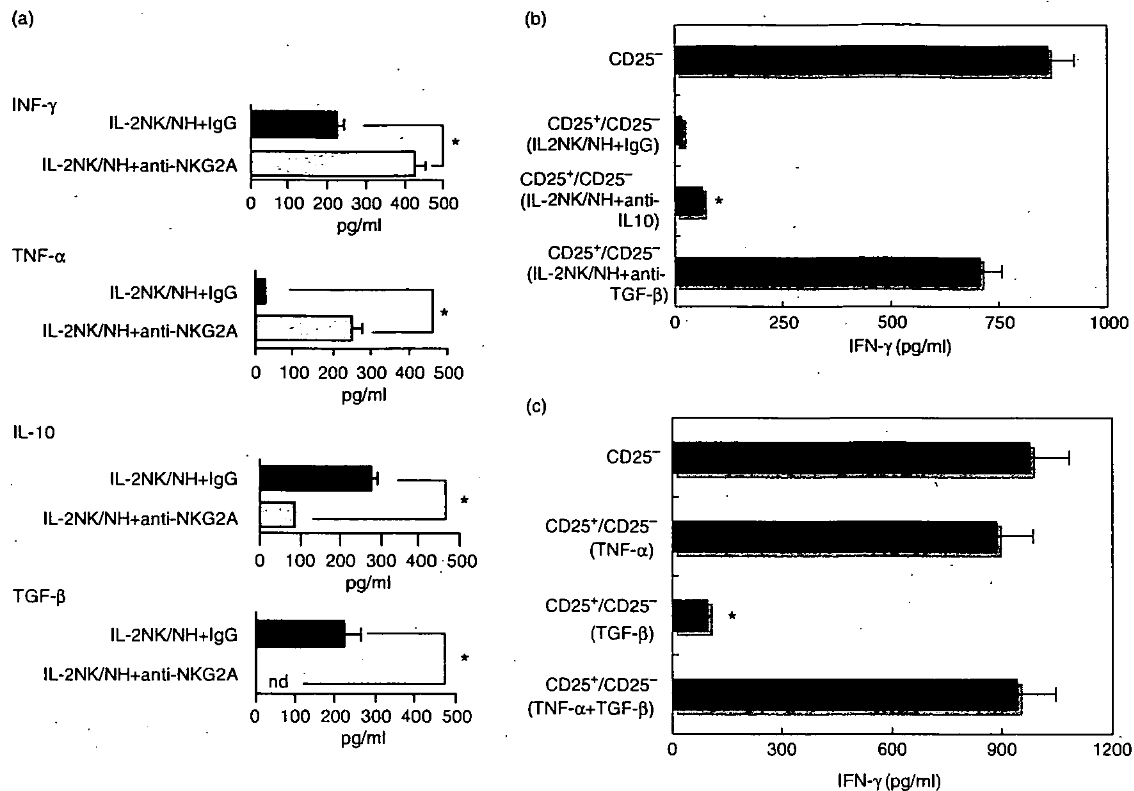

Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the presence of masking antibodies (Abs) of NKG2A (IL-2 NK/NH + anti-NKG2A) or isotype control IgG (IL-2 NK/NH + IgG) for 24 hr. $^*P < 0{\cdot}05$. (b) IL-2 activated NK cells were co-cultured with NHs (IL-2 NK/NH). DCs (1×10^5) were stimulated with the culture supernatant in the presence of anti-IL-10, anti-transforming growth factor (TGF)-β neutralizing Ab or control IgG for 24 hr. DCs were washed thoroughly and co-cultured with allogeneic $CD4^+$ T cells for 48 hr. Next, the isolated $CD4^+$ $CD25^+$ T cells (1×10^5/well) were co-cultured with autologous $CD4^+$ $CD25^-$ T cells in the presence of plate-bound anti-CD3 Ab at a ratio of 1 : 1. Interferon (IFN)-γ production from the culture supernatant was examined by enzyme-linked immunosorbent assay. $^*P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (c) DCs (1×10^5) were stimulated with 50 ng/ml TNF-α, 100 ng/ml TGF-β or both for 24 hr. After thorough washing, they were co-cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ T cells (1×10^5/well) were isolated from the DC and $CD4^+$ co-cultures and cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab. IFN-γ production was examined as described above. $^*P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells.

during co-cultures of $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cells in the presence of anti-CD3 Ab. In case of natural $CD4^+$ $CD25^+$ T cells, their suppressive action was partially reversed on addition of anti-CTLA-4 Ab. By contrast, they preserved their suppressive capacity even in the presence of the blocking Ab of GITR, PD-1, TGF-β or IL-10 (Fig. 4a). When $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were used instead of natural $CD4^+$ $CD25^+$ T cells, their suppressive activity was markedly reduced on addition of the blocking Ab of PD-1 but not CTLA-4, IL-10, TGF-β or GITR (Fig. 4a). The regulatory functions of these Treg cells were required for direct cell-to-cell contact because separation of $CD4^+$ $CD25^+$ Treg cells and $CD4^+$ $CD25^-$ T cells in transwell chambers virtually abolished their suppressive effects (data not shown). We also confirmed the presence of PDL-1 expression on $CD4^+$ $CD25^-$ T cells when they were activated with anti-CD3 Ab (Fig. 4b), suggesting that effector cells themselves induce suppressive activities of $CD4^+$ $CD25^+$ Treg cells. Taken together, these results further reinforced the hypothesis that $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were different from natural $CD4^+$ $CD25^+$ Treg cells in their PD-1-dependent suppressive functions.

Discussion

Recent studies have revealed that activated NK cells positively regulate DC activation and maturation either through direct contact via NK cell receptors (NKp30, NKG2D, etc.) or in co-ordination with various kinds of cytokines (IFN-γ, TNF-α, etc.).[15–18] However, the issue of

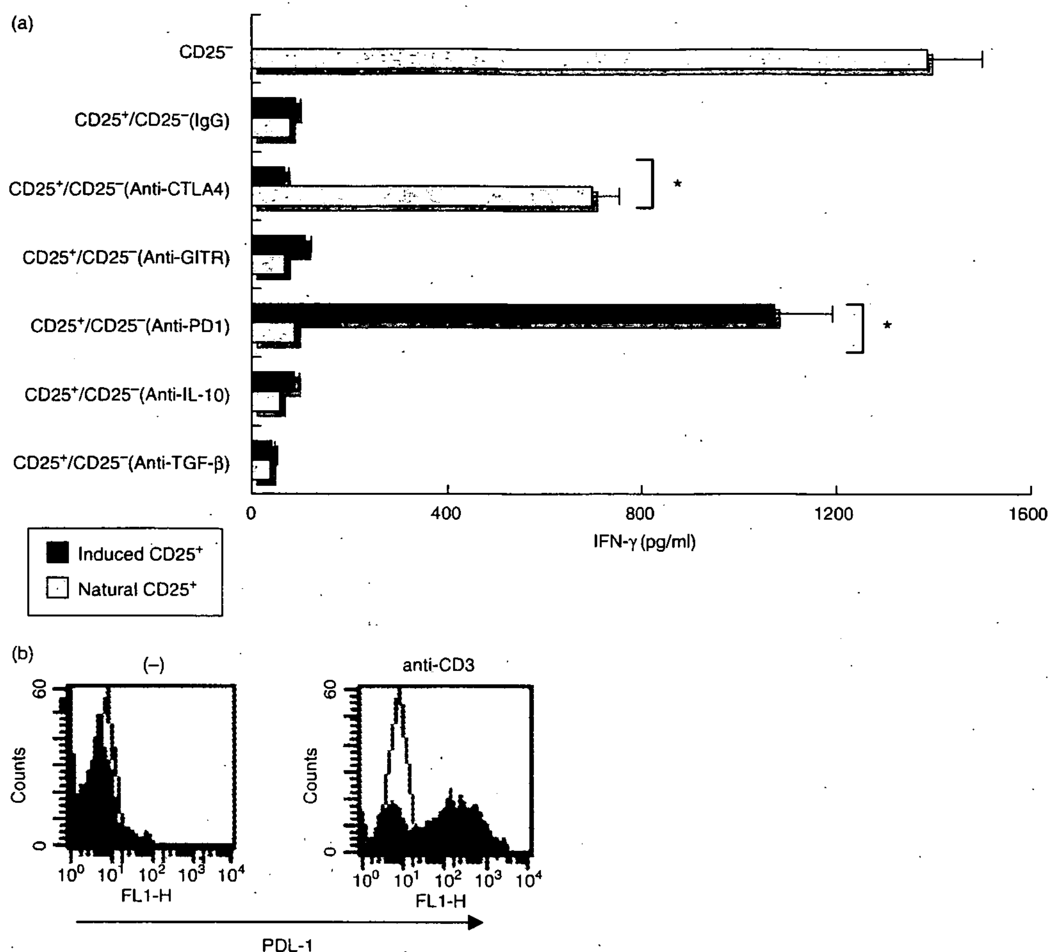

Figure 4. $CD4^+$ $CD25^+$ Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs (1×10^5) were stimulated with the IL-2 NK/NH supernatant for 24 hr, and then cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ fractions were isolated from the DC/$CD4^+$ T cell mixtures. Freshly isolated $CD4^+$ $CD25^+$ T cells (natural $CD25^+$) or $CD4^+$ $CD25^+$ T cells induced by NK/NH-primed DCs (induced $CD25^+$) were co-cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 upon stimulation of plate-bound anti-CD3 antibody (Ab). Anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) Ab, anti-GITR (glucocorticoid-induced TNF receptor) Ab, anti-PD-1 Ab, anti-IL-10 Ab, anti-TGF-β Ab or isotype control IgG (20 μg/ml for each) were incubated during $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cell co-cultures. Interferon (IFN)-γ was measured for each supernatant obtained after 72 hr of co-culture by enzyme-linked immunosorbent assay. *$P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (b) Freshly isolated $CD4^+$ $CD25^-$ T cells were incubated with (anti-CD3) or without (–) plate-bound anti-CD3 Ab for 24 hr. PDL-1 expression was assessed by flow cytometry (closed histograms). Open histograms show isotype control staining.

whether NK cells are involved in DC-mediated Treg cell induction has not been resolved. In the present study, we report that the expression of regulatory markers and functions was markedly decreased on $CD4^+$ $CD25^+$ T cells upon exposure to IL-2 NK-primed DCs. By contrast, the interaction of activated NK cells and NH through the NKG2A inhibitory receptor led to DC induction of $CD4^+$ $CD25^+$ T cells with regulatory properties. Furthermore, NKG2A-mediated increase in TGF-β as well as decrease in TNF-α in an NH and NK cell mixture contributed to DC induction of $CD4^+$ $CD25^+$ Treg cells. This is consistent with previous reports showing that TGF-β plays a role in generating the specific DC that activates $CD4^+$ $CD25^+$ Treg cells.[10,11] The findings that TNF-α suppressed TGF-β-mediated priming of DCs to induce Treg cells also extended the previously identified role of TNF-α as a positive regulator of DC activation. In line with our findings, previous reports showed that impairment of $CD4^+$ $CD25^+$ Treg cell activities restored their suppressive functions after blocking TNF-α signals in non-obese diabetic (NOD) mice or in patients with Crohn's disease.[28,29] To our knowledge, the present study is the first description of modulation of NK cells and human hepatocytes through NKG2A-mediated inhibitory

 © 2006 Blackwell Publishing Ltd,

signals that profoundly affect DC functions towards $CD4^+$ $CD25^+$ Treg cells. Because NK cell functions are regulated by the balance between inhibitory and activating signals, any future clarification of the role of other NK inhibitory and activating receptors in DC modulation and Treg cell activation will be of great interest.

The cross-presentation of self-antigens by major histocompatibility complex (MHC) class II pathways constitutes an important step towards generating and/or expanding peripheral Treg cells.[30] However, we initially settled our experimental design by using DCs and Treg cells from different donors, and DCs encountered $CD4^+$ T cells in an 'antigen-free' condition. Therefore, Treg cells induced by NK/NH-primed DCs are generated independently of MHC class II-mediated self-antigen recognition. These results give rise to the possibility that the cross-talk of NK cells, DCs and hepatocytes represents an alternative pathway in the generation and expansion of peripheral Treg cells. However, it should be noted that these results may not apply to all donors because of the complexity of the allogeneic system and the relatively few donors tested.

PD-1-mediated suppressive activities were characteristic for $CD4^+$ $CD25^+$ Treg cells generated by NH/IL-2 NK-primed DCs. By contrast, natural $CD4^+$ $CD25^+$ Treg cells exerted their suppressive function, at least in part, in a CTLA-4-dependent fashion. Recent reports have clarified the existence of two subtypes of Treg cells: natural and inducible $CD4^+$ $CD25^+$ Treg cells. Inducible Treg cells exert suppressive activities by using molecular mechanisms distinct from those of natural regulatory cells.[31] Our findings further identify the novel pathways by which inducible $CD4^+$ $CD25^+$ Treg cell activities triggered by NKG2A inhibitory signals are dependent on PD-1-mediated negative costimulation. A recent report identified the interaction of B7 on effector T cells with costimulatory molecules CD28/CTLA-4 on $CD4^+$ $CD25^+$ Treg cells as molecular mechanisms of their suppressor activity.[32] Thus, it is possible that reverse signalling of PDL-1 on effector cells may also be crucial for the negative costimulator-mediated suppressive action of $CD4^+$ $CD25^+$ Treg cells. In the present study, we did not address the mechanisms by which NH/IL-2 NK-primed DCs induce $CD4^+$ $CD25^+$ Treg cells with PD-1-dependent suppressive functions. Further study will be needed to clarify this issue.

We previously showed that NKG2A is expressed at higher levels from NK cells isolated from peripheral blood in patients with chronic hepatitis C virus (HCV) infection than from those in healthy donors.[20] HCV frequently persists in humans, at least in part, due to inefficient induction of NK activity as well as specific T cell responses.[33–35] The small percentage of patients who spontaneously clear the virus and recover from chronic hepatitis C mount vigorous HCV-specific $CD4^+$ and $CD8^+$ T cell responses.[36,37] Research has described an increased frequency of $CD4^+$ $CD25^+$ T cells in the blood of patients with persistent HCV infection compared with those who have spontaneously cleared HCV.[38,39] Our current findings raise the interesting possibility that increased NKG2A expression on NK cells may lead to DC-mediated induction of Treg cells, leading to the inhibition of adaptive responses to HCV and failure to eliminate this virus. Indeed, $CD4^+$ $CD25^+$ T cells induced by HCV-NK/Hep3B hepatoma cell-primed DCs expressed and suppressed effector T cell functions at greater levels than those induced by N-NK/Hep3B-primed DCs (our unpublished data). Interestingly, a recent study identified PD-1-mediated signals as a critical pathway to induce anergic $CD8^+$ T cells and impair antiviral CTL responses in chronic viral infection.[40] In this regard, the therapeutic modification of the PD-1 pathway may synergistically augment antiviral immunity by suppressing Treg activity and recovering CTL responses. It is important to establish whether the PD-1 pathway in liver lymphocytes may be operable *in vivo* and play a critical role in suppression of virus-specific immunity in HCV infection.

In conclusion, we have demonstrated that interaction of NK cells and hepatic cells via NKG2A leads to DC induction of $CD4^+$ $CD25^+$ T cells with PD-1-dependent regulatory activities. These findings also imply that NK receptor signals of NK cells may dictate DC-mediated adaptive immune responses towards tolerogenic or immunogenic status via induction of Treg cells.

Acknowledgements

This work was supported by a grant-in-aid from the Ministry of Culture, Sports, Science and Technology of Japan and a grant-in-aid for research on hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan. It was also partially supported by the 21st Century Centre of Excellence Programme of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Shevach EM. $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; 2:389–400.
2 Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification of functional characterization of human $CD4^+$ $CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; **193**:1285–94.
3 Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. *Ex vivo* isolation and characterization of $CD4^+$ $CD25^+$ T cells with regulatory properties from human blood. *J Exp Med* 2001; **193**:1303–10.
4 Belkald Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. $CD4^+$ $CD25^+$ regulatory T cells control Leishmania major persistence and immunity. *Nature* 2002; **420**:502–7.
5 Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, Wang RF. Tumour-specific human $CD4^+$ regulatory T cells

and their ligands: implication for immunotherapy. *Immunity* 2004; **20**:107–18.

6 Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD4^+$ $CD25^+$ regulatory cells that control intestinal inflammation. *J Exp Med* 2002; **192**:295–302.

7 Herman AE, Freeman GJ, Mathis D, Benoist C. $CD4^+$ $CD25^+$ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J Exp Med* 2004; **199**:1479–89.

8 Khoury SJ, Sayegh MH. The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity* 2004; **20**:529–38.

9 Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4 ($^+$) CD25 ($^+$) suppressor T cells *in vivo*. *Nat Immunol* 2004; **3**:33–41.

10 Steinman RM, Hawiger D, Nussenzweig D. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003; **21**:685–711.

11 Mahnke K, Quan Y, Knop J, Enk AH. Induction of $CD4^+$ $CD25^+$ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* 2003; **101**:4862–9.

12 Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. $CD4^+$ $CD25^+$ T cells regulate the expansion of peripheral $CD4^+$ T cells through the production of IL-10. *J Immunol* 2001; **166**:3008–18.

13 Yamagiwa S, Gray JD, Hashimoto H, Horwitz DA. A role of TGF-β in the generation and expansion of $CD4^+$ $CD25^+$ regulatory T cells from human peripheral blood. *J Immunol* 2001; **166**:7282–9.

14 Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF-β regulates *in vivo* expansion of Foxp3-expressing $CD4^+$ $CD25^+$ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci USA* 2004; **101**:4572–7.

15 Moretta A. The dialogue between human natural killer cells and dendritic cells. *Curr Opin Immunol* 2005; **17**:306–11.

16 Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural killer cells and dendritic cells: 'L'union fait la force'. *Blood* 2005; **106**:2252–8.

17 Mocikat R, Braumuller H, Gumy A *et al.* Natural killer cells activated by MHC^{LOW} targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity* 2003; **19**:561–9.

18 Van den Broeke LT, Daschbach E, Thomas EK, Andringa G, Berzofsky JA. Dendritic cell-induced activation of adaptive and innate antitumour immunity. *J Immunol* 2003; **171**:5842–52.

19 Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. *Nat Rev Immunol* 2001; **1**:41–9.

20 Jinushi M, Takehara T, Tatsumi T *et al.* Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to the altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J Immunol* 2004; **173**:6072–81.

21 Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol* 1998; **160**:4951–60.

22 Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999; **285**:727–9.

23 Pende D, Sivori S, Accame L *et al.* HLA-G recognition by human natural killer cells. Involvement of CD94 both as inhibitory and as activating receptor complex. *Eur J Immunol* 1997; **27**:1875–80.

24 Valerie V, Vosters O, Beuneu C, Nicaise C, Stordeur P, Goldman M. Induction of FOXP3-expressing regulatory $CD4^+$ T cells by human mature autologous dendritic cells. *Eur J Immunol* 2003; **34**:762–72.

25 Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of $CD4^+$ $CD25^+$ regulatory T cells through GITR break immunological self-tolerance. *Nat Immunol* 2002; **3**:135–42.

26 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**:1057–61.

27 Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med* 2002; **195**:335–41.

28 Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF-α therapy. *J Exp Med* 2004; **200**:277–85.

29 Wu AJ, Hua H, Munson SH, McDevitt HO. Tumour necrosis factor-α regulation of $CD4^+$ $CD25^+$ T cell levels in NOD mice. *Proc Natl Acad Sci USA* 2002; **99**:12287–92.

30 Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 2005; **6**:1219–27.

31 Bluestone JA, Abbas AK. Natural and adaptive regulatory T cells. *Nat Rev Immunol* 2003; **3**:253–7.

32 Paust S, Lu L, McCarty N, Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci USA* 2004; **101**:10398–403.

33 Ahmad A, Alvarez F. Role of NK and NKT cells in the immunopathogenesis of HCV-induced hepatitis. *J Leukoc Biol* 2004; **76**:743–59.

34 Golden-Mason L, Rosen HR. Natural killer cells: primary target for hepatitis C virus immune evasion strategies. *Liver Transplant* 2006; **12**:363–72.

35 Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005; **5**:215–29.

36 Lauer GM, Barnes E, Lucas M *et al.* High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004; **127**:924–36.

37 Cox AL, Mosbruger T, Lauer GM, Pardoll D, Thomas DL, Ray S. Comprehensive analysis of $CD8^+$ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology* 2005; **42**:104–12.

38 Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for $CD4^+$ $CD25^+$ regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**:1062–71.

39 Rushbrook SM, Ward SM, Unitt E, Vowler M, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress *in vitro* proliferation of virus-specific $CD8^+$ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**:7852–9.

40 Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439**:682–7.

© 2006 Blackwell Publishing Ltd, *Immunology*, **120**, 73–82

JOURNAL OF VIROLOGY, Jan. 2008, p. 196–206
0022-538X/08/$08.00+0 doi:10.1128/JVI.01640-07
Copyright © 2008, American Society for Microbiology. All Rights Reserved.
Vol. 82, No. 1

Lymphocytoid Choriomeningitis Virus Activates Plasmacytoid Dendritic Cells and Induces a Cytotoxic T-Cell Response via MyD88[▽]

Andreas Jung,[1,2] Hiroki Kato,[1] Yutaro Kumagai,[1] Himanshu Kumar,[1] Taro Kawai,[1,3] Osamu Takeuchi,[1,3] and Shizuo Akira[1,3]*

Research Institute for Microbial Diseases, Department of Host Defense, Osaka University, Osaka, Japan[1]; Department of Neonatology, Campus Mitte, Charité-Universitaetsmedizin Berlin, Berlin, Germany[2]; and ERATO, Japan Science and Technology Agency, Osaka, Japan[3]

Received 26 July 2007/Accepted 4 October 2007

Toll-like receptors (TLRs) and retinoic acid-inducible gene I-like helicases (RLHs) are two major machineries recognizing RNA virus infection of innate immune cells. Intracellular signaling for TLRs and RLHs is mediated by their cytoplasmic adaptors, i.e., MyD88 or TRIF and IPS-1, respectively. In the present study, we investigated the contributions of TLRs and RLHs to the cytotoxic T-lymphocyte (CTL) response by using lymphocytoid choriomeningitis virus (LCMV) as a model virus. The generation of virus-specific cytotoxic T lymphocytes was critically dependent on MyD88 but not on IPS-1. Type I interferons (IFNs) are known to be important for the development of the CTL response to LCMV infection. Serum levels of type I IFNs and proinflammatory cytokines were mainly dependent on the presence of MyD88, although IPS-1$^{-/-}$ mice showed a decrease in IFN-α levels but not in IFN-β and proinflammatory cytokine levels. Analysis of *Ifna6*$^{+/GFP}$ reporter mice revealed that plasmacytoid dendritic cells (DCs) are the major source of IFN-α in LCMV infection. MyD88$^{-/-}$ mice were highly susceptible to LCMV infection in vivo. These results suggest that recognition of LCMV by plasmacytoid DCs via TLRs is responsible for the production of type I IFNs in vivo. Furthermore, the activation of a MyD88-dependent innate mechanism induces a CTL response, which eventually leads to virus elimination.

Viral infections are initially recognized by the innate immune system, which eliminates invading viruses by itself and activates an antigen-specific acquired immune response (2, 4, 17). Type I interferons (IFNs) are produced by innate immune cells after virus infection and play a pivotal role in antiviral responses, including apoptosis of virus-infected cells, cellular resistance to viral infection, and activation of natural killer and T cells (15, 25, 34). The expression of type I IFN genes is controlled by intracellular signaling pathways triggered by recognition of viral components with innate pattern recognition receptors. Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), also called RIG-I-like receptors, are two major receptor families responsible for initial viral recognition (20, 28).

TLRs are type I transmembrane receptors responsible for the recognition of microbial components. Among TLRs, TLR7 and TLR9 recognize single-stranded RNA and CpG motif-containing DNA, respectively, and play an important role in virus-induced type I IFN production by plasmacytoid dendritic cells (pDCs) (2, 20). MyD88, a cytoplasmic adaptor protein containing a Toll/interleukin-1 receptor (IL-1R) homology (TIR) domain, is essential for TLR/IL-1R signaling. Mice lacking MyD88 do not respond to various TLR ligands, except for TLR3, a receptor which utilizes TIR domain-containing adaptor-inducing IFN-β (TRIF) in its downstream pathway (2, 40). MyD88 forms a complex with IL-1R-associated kinase 1 (IRAK-1), IRAK-4, and IFN regulatory factor 7 (IRF-7) upon ligand stimulation. Phosphorylated IRF-7 finally translocates into the nucleus to upregulate the expression of a set of IFN-inducible genes (38).

RLHs, including RIG-I and melanoma differentiation-associated gene 5 (MDA5), are cytoplasmic proteins responsible for the recognition of viral double-stranded RNA (20, 41, 42). RIG-I and MDA5 are comprised of caspase recruitment domains (CARDs) and an RNA helicase domain. Studies using knockout mice revealed that RIG-I is responsible for the recognition of various RNA viruses, including vesicular stomatitis virus, influenza virus, Japanese encephalitis virus, and paramyxoviruses such as Newcastle disease virus and Sendai virus, whereas MDA5 detects viruses belonging to the picornavirus family, such as encephalomyocarditis virus (11, 18, 19). RIG-I and MDA5 detect double-stranded RNAs via the helicase domain and initiate downstream signaling cascades via the CARDs by associating with a CARD-containing signaling protein named IFN-β promoter stimulator 1 (IPS-1) (also known as MAVS, VISA, or CARDIF) (21, 27, 33, 39). IPS-1 signals through IκB kinase-related kinases, called IKK-*i* and TBK1, that phosphorylate IRF-3/7 to induce the expression of IFN-inducible genes (2). Cells deficient in IPS-1 fail to produce type I IFNs and NF-κB-dependent proinflammatory cytokines in response to infection with different families of RNA viruses recognized by RIG-I and MDA5 (24, 35). The RLH signaling pathway is critical for type I IFN production in various cell types, including conventional DCs (cDCs), fibroblasts, and

* Corresponding author. Mailing address: WPI Immunology Frontier Research Center, Laboratory of Host Defense, Osaka University, 3-1 Yamada-oka, Suita 565-0871, Osaka, Japan. Phone: 81-6-6879 8303. Fax: 81-6-6879 8305. E-mail: sakira@biken.osaka-u.ac.jp.
▽ Published ahead of print on 17 October 2007.

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

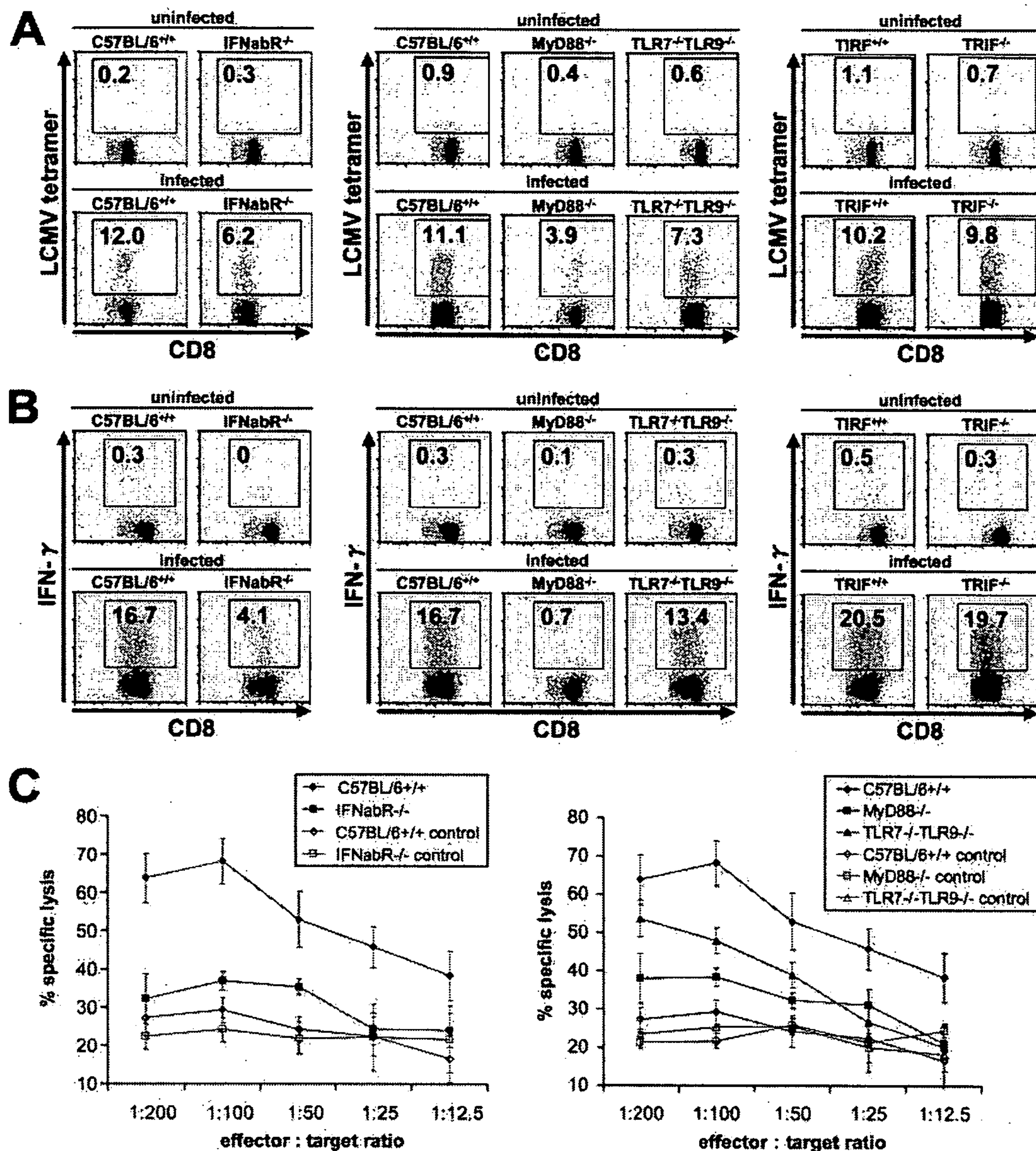

FIG. 1. Induction of LCMV-specific T-cell response via the TLR system. Wild-type, MyD88$^{-/-}$, IFN-α/β receptor$^{-/-}$, TLR7$^{-/-}$ TLR9$^{-/-}$, and TRIF$^{-/-}$ mice were infected intravenously with 3×10^5 PFU LCMV WE, and splenocytes were harvested on day 8 after infection. Cells were stained with LCMV major histocompatibility complex class I tetramer, CD4, and CD8α antibodies (A) or stimulated with gp33-41 prior to CD8α antibody staining (B). Induction of LCMV-specific T lymphocytes and IFN-γ production was analyzed by flow cytometry. (C) Ex vivo CTL activity in splenocytes was determined by a 5-h ^{51}Cr release assay, using gp33-41-loaded EL4 cells as targets. Values are means ± standard deviations (SD) for three mice. Data are representative of three independent experiments.

macrophages, but not in pDCs (23, 24). Thus, both the TLR system and the RLH system participate in virus recognition as well as signal transduction leading to IFN induction. However, the exact mechanisms by which TLRs and RLHs are involved in the development of acquired immune responses have yet to be clarified.

Lymphocytoid choriomeningitis virus (LCMV) is an ambisense single-stranded RNA virus belonging to the family *Arenaviridae*. Numerous strains of this noncytolytic pathogen, such as WE and Armstrong 53b (ARM), cause acute infections in mice, whereas rapidly replicating immunosuppressive variants lead to virus persistence and a general immunosuppression (30). Nonimmunosuppressive LCMV infections result in a profound adaptive immune response that is highlighted by the generation of virus-specific CD4$^+$ and CD8$^+$ T lymphocytes. Activated T cells acquire effector functions, such as IFN-γ production and cytolytic activity, that are responsible for clearing the virus, usually within 7 to 15 days after infection, and eventually lead to a functional T-cell memory (32). Initiation of this specific T-lymphocyte induction is considered to rely on antigen-presenting cells, mainly CD8α$^+$ DCs (3). After capturing the viral antigen, the DCs migrate to lymphoid organs, such as the spleen, where they activate naïve T cells (12). In this process, type I IFNs are believed to play a central role in controlling viral infections such as LCMV infection. They have been shown to act directly on T lymphocytes and to induce

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

massive expansion of antigen-specific CD8$^+$ T cells (1, 7, 22, 32).

Nevertheless, the source of type I IFN in response to LCMV remains controversial, and type I IFN-producing cells are not well characterized. pDCs have been shown to be a major source of type I IFNs in various murine virus infections (6, 8, 9). pDCs were also implicated as the source of IFN-α in the LCMV-infected spleen (29). Conversely, it was reported that production of type I IFNs in response to LCMV infection was not impaired in pDC-depleted mice, questioning the importance of pDCs in T-cell induction in response to LCMV (8). Furthermore, cDCs extracted from mice infected with LCMV were reported to produce high IFN-α levels (10).

In the present study, we investigated the involvement of TLRs and RLHs in the development of antigen-specific CD8$^+$ T cells as well as in the production of type I IFNs in response to LCMV infection by using MyD88-deficient (MyD88$^{-/-}$) and IPS-1$^{-/-}$ mice. The development of cytotoxic T lymphocytes (CTLs) was critically dependent on MyD88 but not on IPS-1. MyD88-deficient mice were revealed to be highly susceptible to LCMV infection. In contrast to previous reports, levels of IFN-α, IFN-β, and proinflammatory cytokines in sera were dependent on the presence of MyD88, whereas IPS-1 deficiency resulted in impaired IFN-α production but otherwise normal cytokine levels. pDCs were the major source of IFN-α in LCMV infection. These results suggest that LCMV activates pDCs via TLRs to produce type I IFNs in vivo and that the activation of an innate mechanism leads to the induction of the CTL response and, eventually, virus elimination.

MATERIALS AND METHODS

Mice. IFN-α/β receptor$^{-/-}$, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR8$^{-/-}$, and IPS-1$^{-/-}$ mice have been described previously (14, 16, 24, 36, 40). TLR7$^{-/-}$ and TLR9$^{-/-}$ mice were crossed to yield double TLR7- and TLR9-deficient mice (13, 14). *Ifna6*$^{+/GFP}$ mice were generated as recently described (23).

LCMV infection and virus titration. LCMV WE and ARM were obtained from T. Otheki (31). For virus propagation, L cells were infected with LCMV and cultured at 37°C for 48 h. Supernatants were diluted in phosphate-buffered saline for infection. Mice were infected intravenously with 3×10^5 PFU LCMV strain WE or ARM after a brief anesthesia with diethyl ether. For analysis of survival rates, mice were infected with 2×10^6 PFU. Virus loads in the spleen and liver were investigated on days 4, 8, and 30 after infection. For virus titration, MC57G cells were inoculated with 10-fold serial dilutions of LCMV-containing supernatants in a 24-well plate, covered with 2% methylcellulose, and incubated at 37°C for 48 h. Cells were then fixed with 4% formalin, permeabilized with 0.5% Triton X, and incubated with 10% fetal bovine serum. After subsequent incubation with murine LCMV immunoglobulin G1 (IgG1; Progen Biotechnik) and anti-mouse IgG–horseradish peroxidase (Amersham Biosciences), plates were stained using an AEC peroxidase substrate kit (Vector Laboratories), and virus plaques were counted for each well.

Induction of specific T-cell response. Splenocytes were harvested 8 days after infection. To investigate the activation of LCMV-specific T lymphocytes, cells were incubated with T-select H-2D^b LCMV tetramer-KAVYNFATC-phycoerythrin (PE) (MBL), CD4-fluorescein isothiocyanate (CD4-FITC), and CD8α-allophycocyanin antibodies (BD Pharmingen). Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar). IFN-γ induction was analyzed by stimulating splenocytes with an LCMV H-2D^b-binding peptide (glycoprotein 33-41 [gp33-41] [KAVYNFATM]; Peptide Institute) and 10 ng/ml murine IL-2 prior to incubation with CD8α antibodies. Cells were then fixed, stained with IFN-γ–FITC antibodies in Perm-Wash solution (BD Pharmingen) according to the manufacturer's recommendation, and assessed by flow cytometry. For determination of cytotoxicity of LCMV-specific T cells, splenocytes were incubated for 5 h with EL-4 target cells that had been loaded with gp33-41 and labeled with ^{51}Cr. The percentage of specific lysis for each sample was calculated as follows: [(sample release − spontaneous release)/(maximal release − spontaneous release)] × 100. To analyze the generation of LCMV-specific memory T lymphocytes, mice were infected with 1×10^5 PFU LCMV WE, and splenocytes were analyzed for activation of specific T memory cells and IFN-γ induction as described below.

Cytokine and type I IFN production. IL-1β, IL-6, IL-10, IL-12(p40), IL-12(p70), RANTES, and tumor necrosis factor alpha were measured in sera 24 and 48 h after LCMV infection by a multiplex bead-based flow cytometry assay (Bio-Plex cytokine assay; Bio-Rad Laboratories). IFN-α and IFN-β were determined repeatedly between 12 and 96 h by enzyme-linked immunosorbent assay (PBL Biomedical Laboratories).

Expression of green fluorescent protein (GFP) in splenic DCs and macrophages. Twenty-four and 48 h after LCMV infection, spleens of *Ifna6*$^{+/GFP}$ mice were injected with 150 U/ml collagenase buffer (Wako Chemicals), 10 μg/ml DNase I (Sigma), and 10% fetal calf serum and incubated for 40 min at 37°C. After the addition of 10 mM EDTA and incubation for another 5 min, spleens were shredded and passed through a nylon mesh. Erythrocytes were lysed, and the single-cell suspension was stained with CD11b-PE, B220-PerCP, and CD11c-allophycocyanin antibodies before fluorescence-activated cell sorter analysis.

Activation of splenic DCs. Splenocytes were harvested 48 h after infection, incubated with B220-PerCP, CD11c-FITC, and CD40-, CD80-, or CD86-PE antibodies (BD Pharmingen), and analyzed by flow cytometry.

TABLE 1. Total and virus-specific numbers of CD8$^+$ T lymphocytes per spleen for uninfected mice and mice that were intravenously infected with 3×10^5 PFU LCMV WE[a]

Mouse group and genotype	Total CD8$^+$ cells	Specific CD8$^+$ cells	% of total
Uninfected mice			
C57BL/6$^{+/+}$	6.6 ± 2.6	0.03 ± 0.04	0.5
IFNabR$^{-/-}$	5.4 ± 0.3	0.03 ± 0.01	0.6
MyD88$^{-/-}$	7.1 ± 1.1	0.03 ± 0.01	0.4
TLR7$^{-/-}$ TLR9$^{-/-}$	6.7 ± 2.2	0.02 ± 0.02	0.3
Infected mice			
C57BL/6$^{+/+}$	19.7 ± 9.2	2.19 ± 1.04	11.1
IFNabR$^{-/-}$	15.7 ± 4.1	0.99 ± 0.25	6.3
MyD88$^{-/-}$	3.6 ± 0.7	0.16 ± 0.06	4.4
TLR7$^{-/-}$ TLR9$^{-/-}$	13.0 ± 2.7	0.98 ± 0.23	7.5

[a] Splenocytes were harvested on day 8 after infection and stained with LCMV major histocompatibility complex class I tetramer and CD8α antibodies. Cell numbers were counted by flow cytometry. Depicted values are multipliers of 10^6 and indicate means ± standard deviations for nine mice per indicated genotype.

RESULTS

Impaired CTL response to LCMV infection in MyD88$^{-/-}$ mice but not IPS-1$^{-/-}$ mice. LCMV has been shown to mount a robust CTL response in vivo. Although the involvement of type I IFNs and innate immune cells in activating CTL responses has clearly been demonstrated before, the mechanism of innate recognition of LCMV in vivo is not yet fully understood. Therefore, we first examined the contributions of two major innate viral recognition systems, TLRs and RLHs, to mounting CTL responses against LCMV infection. Eight days after intravenous administration of LCMV, splenocytes of mice were harvested and stained with an H-2D^b LCMV-specific tetramer (Fig. 1A) or stimulated with gp33-41 for analysis of IFN-γ production (Fig. 1B), and total and virus-specific CD8$^+$ T lymphocytes per spleen were counted (Table 1). To assess LCMV-specific CTL activity, cells were incubated with gp33-41-loaded, ^{51}Cr-labeled EL-4 target cells and specific lysis was calculated (Fig. 1C). Wild-type mice mounted a vigorous CTL response, demonstrated by massive expansion of CD8$^+$ T cells, significant induction of LCMV-specific CD8$^+$ T cells, strong IFN-γ production,

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

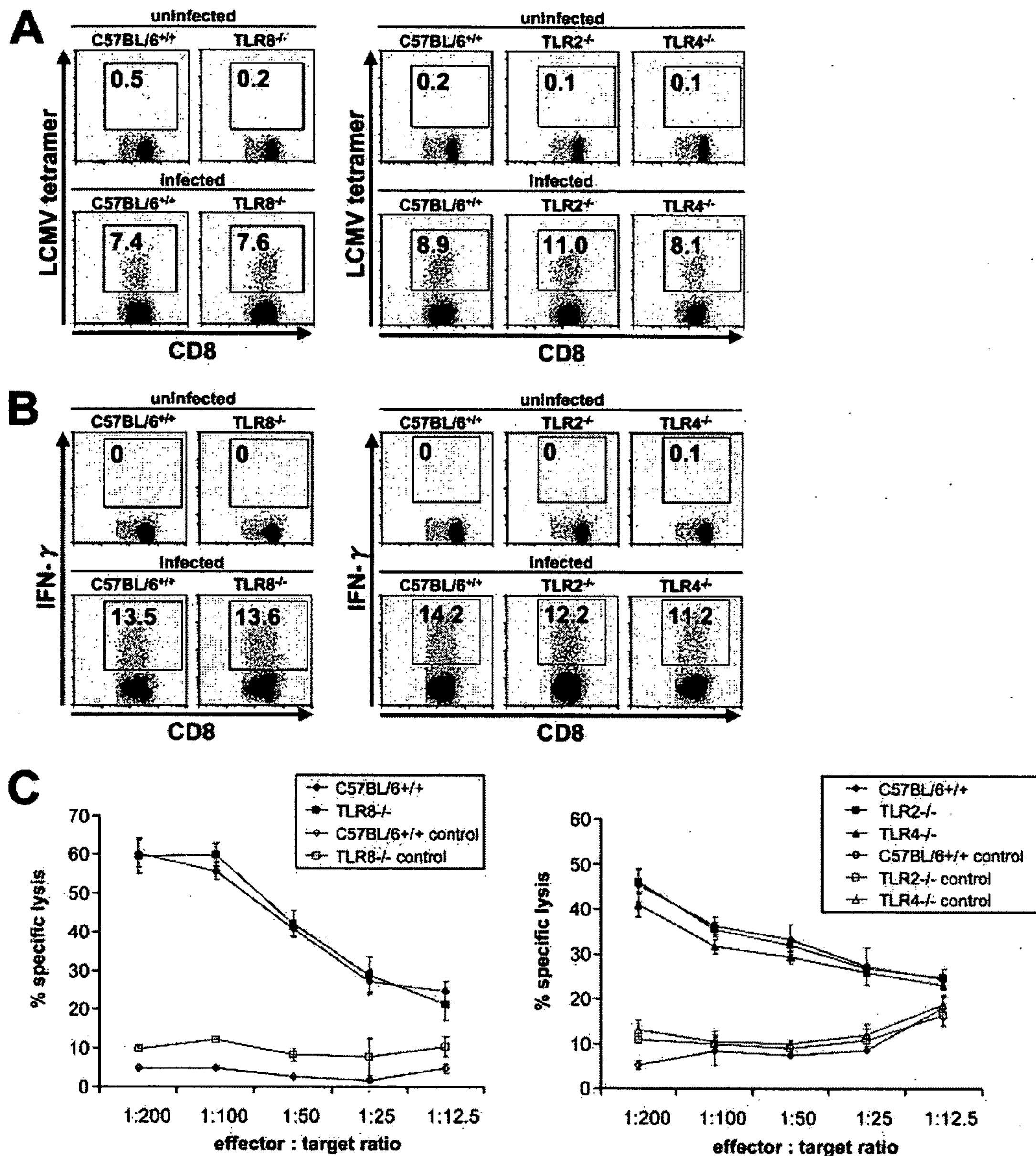

FIG. 2. Involvement of other TLRs in T-cell response. Wild-type, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR8$^{-/-}$ mice were infected intravenously with 3×10^5 PFU LCMV WE, and splenocytes were harvested on day 8 after infection. Induction of LCMV-specific T lymphocytes (A), IFN-γ production (B), and CTL activity (C) were analyzed. Values are means ± SD for three mice.

and specific, T-cell-induced lysis of >60% of labeled target cells. IFN-α/β receptor$^{-/-}$ mice and TLR7$^{-/-}$ TLR9$^{-/-}$ mice demonstrated diminished clonal expansion of cytotoxic T cells in response to LCMV infection, whereas DC8$^+$ T cells even decreased in MyD88$^{-/-}$ mice during infection compared to those in uninfected controls, resulting in splenic atrophy. Consistent with previous reports, IFN-α/β receptor$^{-/-}$ mice also showed impaired induction of LCMV-specific T cells as well as decreased IFN-γ production by these cells. Furthermore, cytotoxic T cells derived from IFN-α/β receptor$^{-/-}$ mice failed to effectively lyse target cells that presented an LCMV-specific epitope. MyD88 proved to play a crucial role in this process, as specific T-cell induction, IFN-γ production, and cytotoxicity were severely affected in MyD88$^{-/-}$ mice. In contrast, TRIF$^{-/-}$ mice did not show a defect in the activation of CD8$^+$ T cells, suggesting that TLR3 is not involved in LCMV-induced CTL responses. When mice lacking various TLRs were infected with LCMV, TLR7$^{-/-}$ TLR9$^{-/-}$ mice showed slightly diminished activation of CTL responses. In contrast, TLR2, TLR4, and TLR8 were not involved in the induction of CTL activity (Fig. 2A to C).

Next, we investigated the role of RLHs in LCMV-induced CD8$^+$ T-cell activation. The analysis of IPS-1$^{-/-}$ splenocytes revealed a normal T-cell response after LCMV infection. Specific T-cell activation, IFN-γ production, and specific lysis were comparable to those of wild-type cells (Fig. 3), suggesting that RLHs are not involved in adaptive immunity after LCMV

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

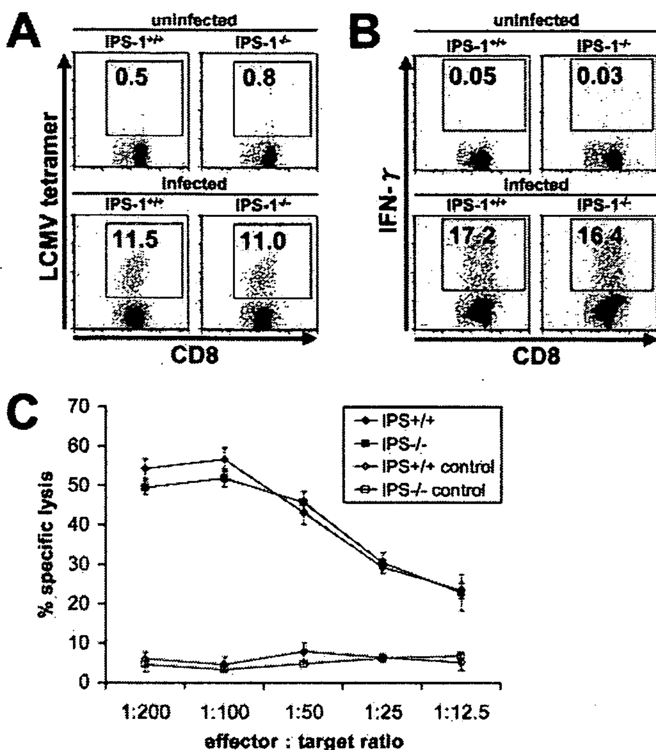

FIG. 3. T-cell response via the RLH system. Wild-type and IPS-$1^{-/-}$ mice were infected intravenously with 3×10^5 PFU LCMV WE, and splenocytes were harvested on day 8 after infection. Induction of LCMV-specific T lymphocytes (A), IFN-γ production (B), and CTL activity (C) were analyzed as described in the legend to Fig. 1. Values are means ± SD for three mice. Data are representative of three independent experiments.

infection. In general, the results obtained herein showed no difference between the WE and ARM strains of LCMV (data for LCMV ARM are not shown).

Role of TLRs and RLHs in the production of type I IFN and proinflammatory cytokines in response to LCMV infection. We further investigated how the TLR system contributes to the activation of CTL responses. Given the importance of type I IFN receptors in the development of LCMV-induced T-cell activity, we hypothesized that MyD88 is involved in the production of type I IFNs, although previous reports have shown that IFN-α production is not impaired in MyD88$^{-/-}$ mice with LCMV infection. We therefore measured serum levels of IFN-α and cytokines between 12 h and 96 h after intravenous LCMV challenge. In wild-type mice, IFN-α and IFN-β peaked 24 h after infection, and IFN-α levels gradually decreased after 48 h. IFN-α production was severely impaired in MyD88$^{-/-}$ mice (Fig. 4A), and IFN-β production.was abolished (Fig. 4B), suggesting that TLRs play a critical role in type I IFN production in response to LCMV. In contrast, IFN-α production was not impaired in the absence of TRIF (data not shown), showing that TLR3 is not involved in LCMV-induced type I IFN production. Mice deficient in both TLR7 and TLR9 showed a partially impaired type I IFN response. TLR2, TLR4, and TLR8 did not play any role in the IFN response (data not shown). Thus, it is possible that a combination of TLRs might be important for the recognition of whole LCMV via MyD88 in vivo. We then investigated the involvement of RLHs by using IPS-$1^{-/-}$ mice. Although LCMV-induced IFN-α production was modestly impaired (Fig. 4A), IFN-β production was not impaired in IPS-$1^{-/-}$ mice (Fig. 4B), suggesting that the contribution of RLHs to LCMV-induced type I IFN production is smaller than that of TLRs. In general, no strain-specific differences were observed between LCMV WE and ARM infections (data for LCMV ARM are not shown).

Furthermore, we examined the levels of proinflammatory cytokines in sera. The production of IL-6, IL-12(p40), and RANTES was abolished in MyD88$^{-/-}$ mice (Fig. 5A and B). Conversely, IPS-1 deficiency did not significantly alter the production of these cytokines, suggesting that the TLR system, but not RLHs, plays an important role in the production of cytokines.

Identification of IFN-α-producing cells during LCMV infection. Previous reports pointed out that cells other than pDCs are responsible for IFN-α production during LCMV infection. Therefore, we further investigated IFN-α-producing cells in LCMV infection by using *Ifna6*$^{+/GFP}$ reporter mice. We found that 24 and 48 h after intravenous LCMV infection, GFP$^+$ cells were most frequently observed in the spleen (Fig. 6A and B) and, to a lesser extent, in inguinal lymph nodes, bone marrow, and the liver (data not shown). LCMV infection intensively increased the number of GFP$^+$ B220$^+$ CD11c^{dull} pDCs, indicating that they were the major IFN-α producers in response to LCMV infection. LCMV also modestly increased the numbers of GFP$^+$ B220$^-$ CD11c$^+$ cDCs and GFP$^+$ CD11c$^-$ CD11b$^+$ macrophages in the spleen (Fig. 6A and B), although this phenomenon was not observed in other organs (data not shown). Expression of GFP$^+$ pDCs was abolished in the absence of MyD88, whereas no significant difference was observed between wild-type and IPS-$1^{-/-}$ mice, providing evidence that pDCs utilize the TLR system to produce IFN-α. The number of GFP$^+$ cDCs and macrophages was slightly but constantly reduced in *Ifna6*$^{+/GFP}$ reporter mice lacking IPS-1 but was normal in MyD88$^{-/-}$ mice, suggesting that IFN-α production by these cell types in response to LCMV requires the RLH system and is independent of TLRs (Fig. 6A and B). Taken together, these data indicate that pDCs are the main type I IFN producers during LCMV infection through virus recognition by the TLR-MyD88 system.

Activation of DCs in response to LCMV infection. DCs have been shown to be important for the activation of adaptive immunity. One attribute of DC activation is the surface upregulation of costimulatory molecules (29). To analyze whether different DC subsets were activated in response to LCMV infections, we assessed the expression of surface CD80, CD86, and CD40 on splenic pDCs and cDCs 24 and 48 h after infection. In wild-type mice, DC expression of CD80 (Fig. 7A and B), CD86, and CD40 (data not shown) was enhanced after infection. While no differences in the induction of these surface molecules were found between wild-type and MyD88- or IPS-1-deficient cDCs, upregulation was impaired in pDCs in MyD88- but not IPS-1-deficient mice, presenting further evidence that MyD88 plays an important role in the pDC-mediated innate response to LCMV infections.

Enhanced susceptibility of MyD88$^{-/-}$ mice to LCMV infection. When wild-type mice were challenged intravenously with LCMV, the virus was cleared by the activation of CTLs, lead-

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

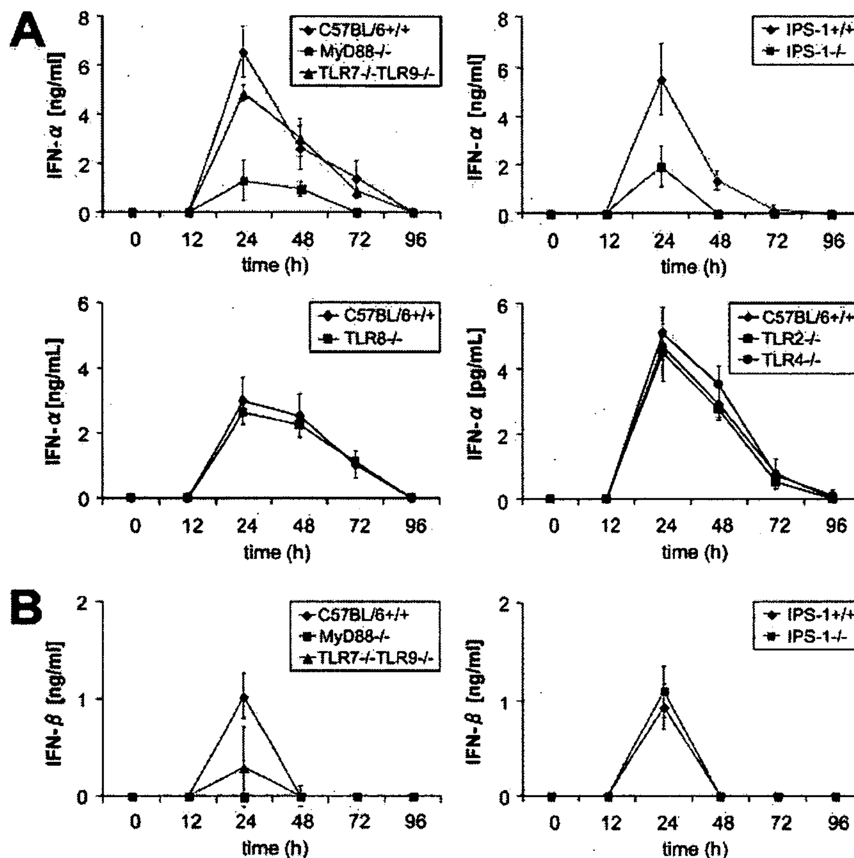

FIG. 4. Type I IFN response to LCMV infection. The indicated mice were infected intravenously with 3×10^5 PFU LCMV WE, and IFN-α (A) and IFN-β (B) levels in sera were measured by enzyme-linked immunosorbent assay between 12 and 96 h after infection. Data show means ± SD for three mice and are representative of three independent experiments.

ing to full recovery. Because MyD88$^{-/-}$ mice failed to mount a sufficient T-cell response, we further investigated the long-term outcome of LCMV infections. As expected, IPS-1$^{-/-}$ mice effectively cleared the virus from the spleen (Fig. 8B) and other organs (data not shown) within 1 week after infection, in a manner similar to that of wild-type mice. In contrast, the absence of MyD88 resulted in a failure of virus elimination, with viral titers persisting for several weeks, independent of TLR7 and TLR9 (Fig. 8A and C). This observation was in accordance with a long-lasting deficiency in T-cell function (Fig. 8E and F). The severe defects in virus clearance and in the generation of LCMV-specific T memory cells were underlined by the finding that MyD88$^{-/-}$ mice died between 30 and 40 days after challenge with higher virus titers (Fig. 8D). These results demonstrate that MyD88 is essential for the survival of mice with LCMV infection by enabling the activation of acquired immune responses.

DISCUSSION

LCMV has been studied intensively to understand the mechanisms of CTL activation following recognition of specific antigens. However, the role of innate immunity in the activation of CTL responses as well as in the elimination of the virus has yet to be clarified. When analyzing mice deficient in MyD88 or IPS-1, we clearly showed that the TLR system, but not the RLH system, is critical for the development of adaptive immunity against LCMV infection. Since the expression of IFN-α/β receptor on antigen-specific CD8$^+$ T cells is important for their expansion, activation, and memory formation after viral infection (22), the contribution of MyD88 to this process seems to be via the initial control of type I IFN production. We demonstrate that both IFN-α and IFN-β are mainly controlled by MyD88-dependent pathways. IFN-α and IFN-β share the IFN-α/β receptor for signaling, and mice deficient in IFN-α/β receptor showed impaired CTL activity during LCMV infection. Therefore, it is presumable that both IFN-α and IFN-β contribute to the generation of a CTL response to LCMV. Notably, it appears that MyD88$^{-/-}$ mice have a more severe defect in CTL responses than do IFN-α/β receptor$^{-/-}$ mice, a phenomenon also observed for CD8$^+$ T memory cells. Furthermore, IPS-1$^{-/-}$ mice did not show a defect in CTL responses, although they showed partially impaired serum IFN-α levels after LCMV infection. Thus, it is possible that the development of LCMV-specific T cells is not solely regulated by type I IFNs. In fact, at least two different pathways for LCMV-induced T-cell IFN-γ production have been described (7). Under normal conditions, the IFN-γ response is dependent on type I IFN, without a contribution of IL-12. However, mice deficient in IFN-α/β receptor elicit elevated levels of IL-12, and this may overcome the defect caused by IFN-α/β deficiency on CD8$^+$ T cells. MyD88 regulates the production of

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

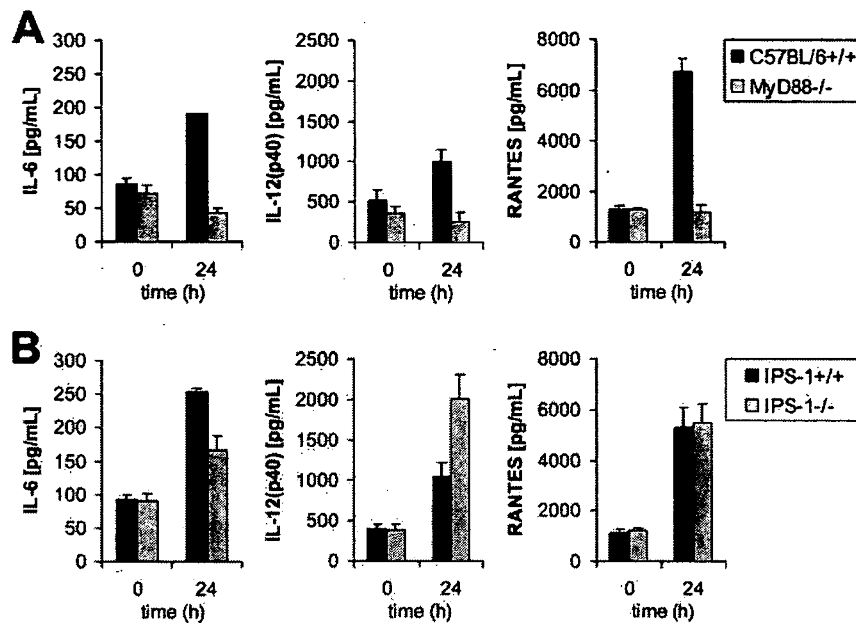

FIG. 5. Cytokine production. IL-6, IL-12(p40), and RANTES levels in sera of MyD88$^{-/-}$ (A) and IPS-1$^{-/-}$ (B) mice after intravenous infection with 3 × 10^5 PFU LCMV WE were assessed after 24 h by a multiplex bead-based assay. Data show means ± SD for three mice.

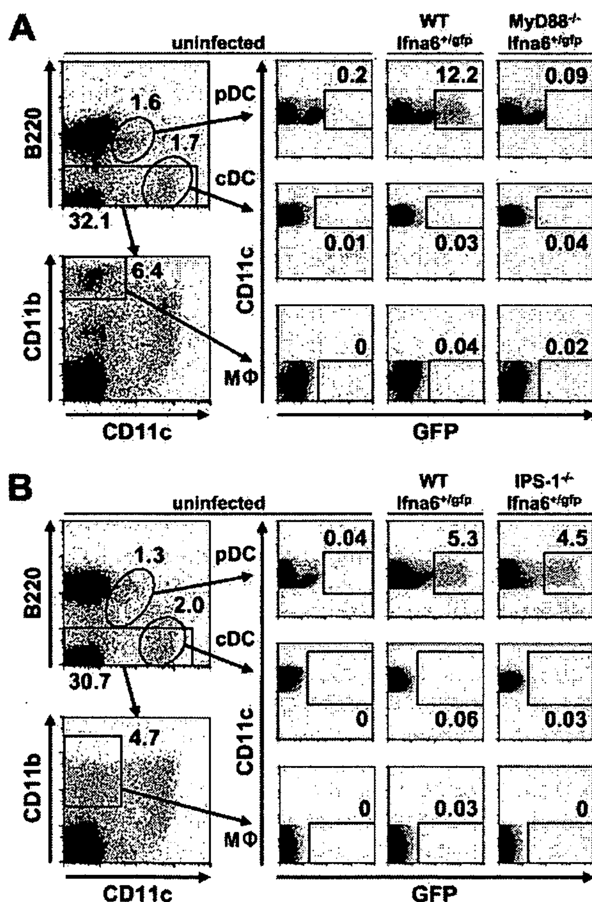

FIG. 6. Expression of GFP in splenic DCs and macrophages. *Ifna6*$^{+/GFP}$ mice lacking MyD88 (A) or IPS-1 (B) as well as wild-type mice were infected intravenously with 3 × 10^5 PFU LCMV WE. Splenocytes were harvested 24 and 48 h after infection, and the expression of GFP in pDCs, cDCs, and macrophages was analyzed by fluorescence-activated cell sorter analysis. Data are representative of two experiments.

various cytokines, such as IL-1, IL-6, IL-12, IL-18, and RANTES, in addition to type I IFNs, and these cytokines likely contribute to the MyD88-dependent development of LCMV-specific T cells in vivo.

Previous reports have shown that the production of type I IFNs in response to LCMV infection is not impaired in pDC-

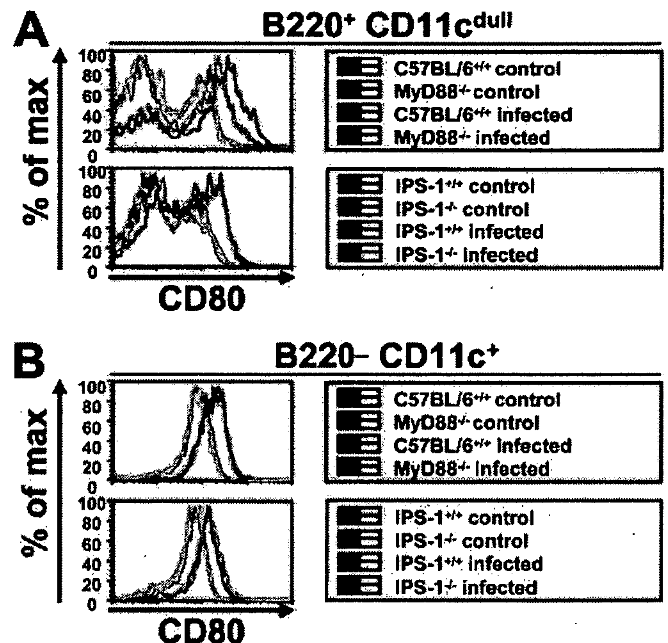

FIG. 7. Activation of splenic DCs. Mice were infected intravenously with 3 × 10^5 PFU LCMV WE. Splenocytes of MyD88- and IPS-1-deficient mice were investigated for the activation of CD80 on pDCs (A) and cDCs (B) by flow cytometry 48 h after infection. Data shown are representative of three mice.

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

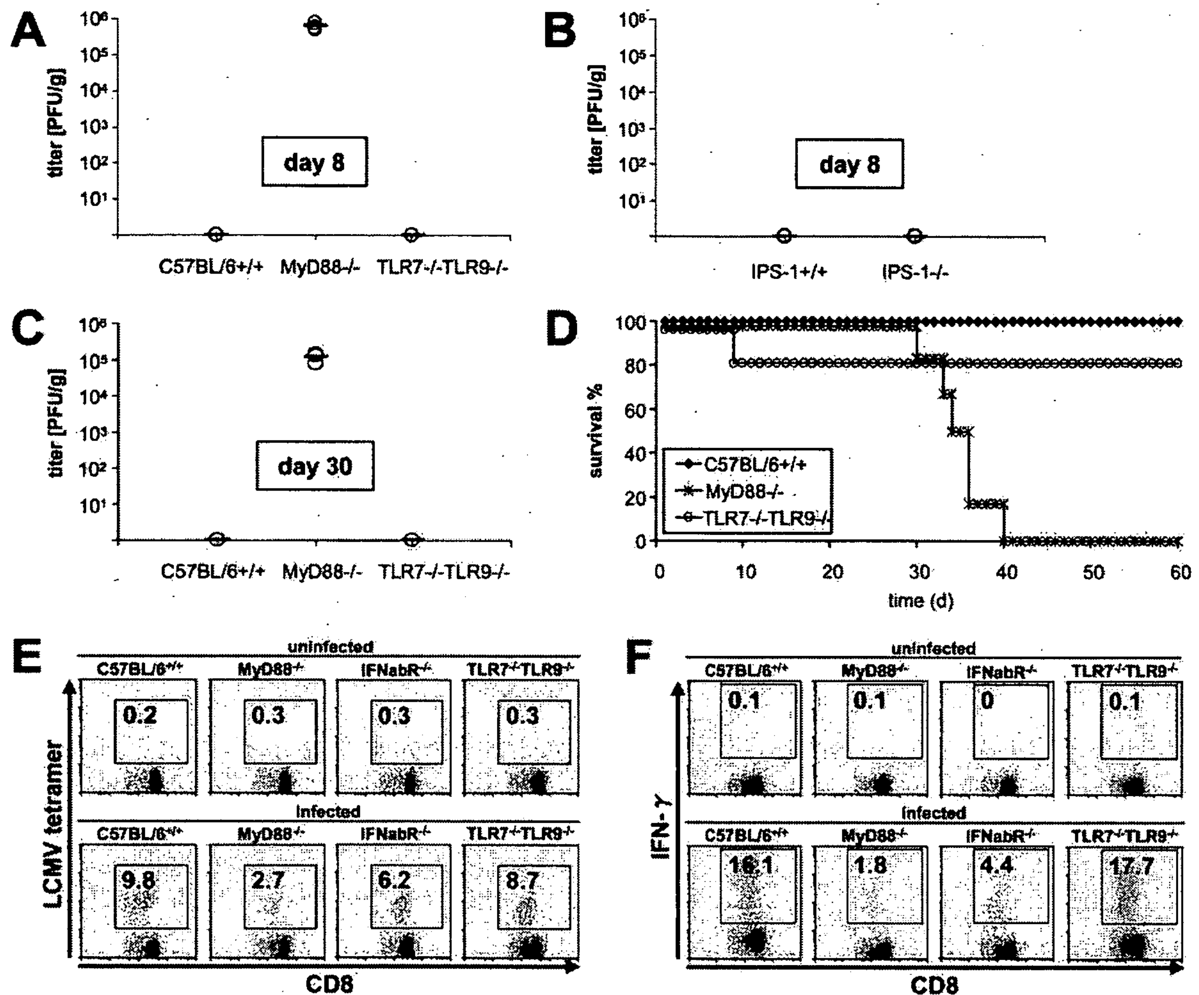

FIG. 8. Long-term sequelae after LCMV infection. Wild-type, MyD88$^{-/-}$, and TLR7$^{-/-}$ TLR9$^{-/-}$ mice or IPS-1$^{-/-}$ mice were infected intravenously with 3×10^5 PFU LCMV WE, and virus titers were measured in spleens on day 8 (A and B) and day 30 (C) by a methylcellulose titration assay. Results for three mice are displayed, with means. Data are representative of three independent experiments. (D) Survival rates were assessed after infecting six mice per indicated genotype with 2×10^6 PFU LCMV WE in two independent experiments. (E and F) Generation of LCMV-specific memory CD8$^+$ T lymphocytes in MyD88$^{-/-}$, TLR7$^{-/-}$ TLR9$^{-/-}$, and IFN-α/β receptor$^{-/-}$ mice following infection with 1×10^5 PFU LCMV WE was analyzed as described above. Indicated values are means ± SD for three mice and are representative of two independent experiments.

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

depleted mice (8) and that splenic cDCs from infected mice produce IFN-α (10). On the other hand, it was also suggested that splenic pDCs can produce IFN-α in response to LCMV infection (29). In this study, we identified pDCs as the predominant IFN-α producers by infecting *Ifna6*$^{+/GFP}$ mice with LCMV. We also observed a modest expression of GFP$^+$ cDCs and macrophages in LCMV-infected reporter mice; however, this expression was clearly subordinate to that of pDCs. Other cell types, such as T and B cells, did not express GFP in early LCMV infection (data not shown), and no type I IFNs could be detected in sera later than 72 h postinfection, indicating that lymphocytes are not IFN-α producers in response to LCMV. Furthermore, we found that IFN-α production in pDCs was exclusively dependent on MyD88 but not on IPS-1. Consistently, IFN-α and IFN-β levels in sera were severely impaired in MyD88$^{-/-}$ mice. These results are in contradiction to a previous report showing that the production of type I IFNs in response to LCMV was MyD88 independent (43). Although we do not have an explanation for this discrepancy, we believe that the involvement of MyD88 as well as pDCs in the IFN response is well grounded based on the data from measurements of serum IFN and our novel reporter mice. On the other hand, cDCs and macrophages from *Ifna6*$^{+/GFP}$ mice lacking IPS-1 showed reduced GFP expression in response to LCMV compared to those from wild-type mice, although the frequency of GFP$^+$ cells was much lower than that for pDCs. However, given that the total number of cDCs and macrophages in the body by far exceeds the number of pDCs, it can be presumed that the impaired IFN-α levels in sera in IPS-1$^{-/-}$ mice are a result of the failure to produce IFN in these cell types.

Since type I IFN production in response to LCMV infection was mainly dependent on MyD88, we investigated the contribution of each TLR to the recognition of LCMV infection. Although the involvement of TLR2 was reported previously (43), we did not detect a defect in TLR2$^{-/-}$, TLR4$^{-/-}$, or

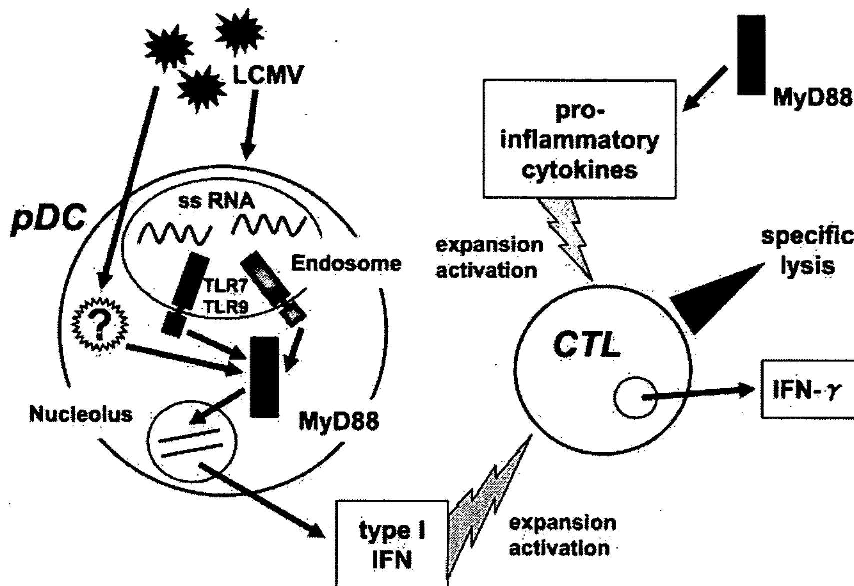

FIG. 9. Innate immune modulation of CTL response to LCMV through MyD88 signaling. MyD88 mediates recognition of LCMV by TLR7/TLR9 and possibly other, unknown receptors via pDCs. Type I IFNs produced by infected pDCs result in activation and clonal expansion of virus-specific CTLs. In an alternative pathway, CTLs are activated by proinflammatory cytokines in an MyD88-dependent manner. Activated CTLs mount effector functions that finally contribute to virus elimination.

TLR8$^{-/-}$ mice. TLR1 and TLR6 both form heterodimers with TLR2 to recognize bacterial components, and they are likely not involved in LCMV recognition, since TLR2$^{-/-}$ mice responded appropriately to LCMV infection. Also, TLR5 is unlikely to contribute to MyD88-dependent LCMV recognition, as it does not activate IFN-inducible genes and is only marginally expressed in splenic cells (37). TLR7$^{-/-}$ TLR9$^{-/-}$ mice showed only partial impairment in type I IFN levels and CTL activity compared to MyD88$^{-/-}$ mice, and TRIF was not involved in innate and adaptive immune responses to LCMV. Taken together, the data show that other unknown MyD88-dependent but TLR7- and TLR9-independent receptors may contribute to the complex orchestration of LCMV signal recognition in vivo. Another possibility is that pDCs are indirectly activated by LCMV-infected cells and that MyD88-dependent signaling is required in other cell types. Indeed, pDCs induced from bone marrow failed to produce large amounts of IFN-α in response to LCMV infection in vitro, whereas infection with Newcastle disease virus, Sendai virus, and influenza virus was reported to induce large amounts of IFN-α in bone marrow-derived pDCs (19). Thus, it is possible that IFN-α production in response to LCMV is mediated through a mechanism different from that in response to other RNA viruses. Further studies are required to clarify the mechanism of IFN-α production in pDCs via the MyD88-dependent pathway.

DCs are reported to be critical for the development of antigen-specific T-cell responses against viral infection (3). In response to LCMV infection, cDCs upregulated costimulatory molecules, namely, CD40, CD80, and CD86, even in the absence of MyD88 or IPS-1. In contrast, costimulatory molecule expression in pDCs was impaired in MyD88-deficient mice. The critical role of MyD88-dependent signaling in CD8 T-cell activation and its role in IFN production in pDCs suggested that pDCs are essential for inducing the development of LCMV-specific CTL responses. However, it is not clear if pDCs play a direct role in the presentation of LCMV antigen to CD8 T cells. A possible explanation is that pDCs act to facilitate CTL activation by producing type I IFN rather than directly presenting antigens. A model depicting the modulation of the CTL response to LCMV by pDCs via MyD88 is shown in Fig. 9.

MyD88-deficient mice were highly susceptible to infection with LCMV and showed long-lasting virus persistence. It seems that the defect in the acute-phase innate response cannot explain the cause of death observed for MyD88$^{-/-}$ mice, considering that all MyD88-deficient mice died after 30 to 40 days of infection with higher virus titers. These results are similar to the effects of LCMV infection in perforin-deficient (Perf$^{-/-}$) mice. Perf$^{-/-}$ mice have been shown to enhance T-cell expansion and activation in response to persistent LCMV infection, leading to immunomediated tissue damage and increased mortality (5, 26). Thus, it has been suggested that the pathology and fatality of LCMV infection are most likely not direct results of the infection but, rather, are due to virus-induced immunopathology. Therefore, although LCMV-mediated CTL responses, including the formation of CD8α^{+} T memory cells, were impaired in MyD88$^{-/-}$ mice, the persistent T-cell response may eventually have caused enough pathology to result in the death of the mice. However, further studies are required to clarify the precise cause of death observed for MyD88$^{-/-}$ mice.

In summary, our results demonstrate the importance of MyD88-dependent signaling in the production of type I IFNs in pDCs. Consistently, MyD88 is critical for the activation of CTL responses. Furthermore, MyD88 controls various proinflammatory cytokines that are likely to contribute to the activation of CTL responses independently of type I IFN. However, the cell-specific contributions of TLRs and cytoplasmic

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

RLHs to the activation of acquired immunity appear to be different in response to various viruses. Future studies will clarify how these two innate viral recognition pathways are involved in T-cell activation depending on different viral pathogens.

ACKNOWLEDGMENTS

We thank Y. Fujiwara, M. Shiokawa, and A. Shibano for skillful technical assistance, N. Kitagaki for an excellent methodological tutorial, and P. Lee for critically reading the manuscript. M. Hashimoto deserves special appreciation for distinguished organizational support and secretarial assistance. T. Otheki kindly provided L cells and MC57G cells. EL4 cells were gratefully obtained from H. Tsutsui.

This work was supported in part by grants from the Yokochi Fund of the Kanehara Ichiro Foundation, from the Ministry of Education, Culture, Sports, Science and Technology in Japan, from the 21st Century Center of Excellence Program of Japan, and from the NIH (AI070167).

We have no competing financial interests.

REFERENCES

1. **Aichele, P., H. Unsoeld, M. Koschella, O. Schweier, U. Kalinke, and S. Vucikuja.** 2006. Cutting edge: CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. J. Immunol. **176:**4525–4529.
2. **Akira, S., S. Uematsu, and O. Takeuchi.** 2006. Pathogen recognition and innate immunity. Cell **124:**783–801.
3. **Belz, G. T., K. Shortman, M. J. Bevan, and W. R. Heath.** 2005. CD8α dendritic cells selectively present MHC class I-restricted noncytological viral and intracellular bacterial antigens in vivo. J. Immunol. **175:**196–200.
4. **Beutler, B.** 2004. Interferences, questions and possibilities in Toll-like receptor signaling. Nature **230:**257–263.
5. **Binder, D., M. F. van den Broek, D. Kaegi, H. Bluethmann, J. Fehr, H. Hnegartner, and R. M. Zinkernagel.** 1989. Aplastic anemia rescued by exhaustion of cytokine-secreting CD8$^+$ T cells in persistent infection with lymphocytic choriomeningitis virus. J. Exp. Med. **187:**1902–1920.
6. **Colonna, M., A. Krug, and M. Cella.** 2002. Interferon-producing cells: on the front line in immune responses against pathogens. Curr. Opin. Immunol. **14:**373–379.
7. **Cousens, L. P., R. Peterson, S. Hsu, A. Dorner, J. D. Altman, R. Ahmed, and C. A. Biron.** 1999. Two roads diverged: interferon α/β- and interleukin 12-mediated pathways in promoting T cell interferon γ responses during viral infection. J. Exp. Med. **189:**1315–1327.
8. **Dalod, M., T. P. Salazar-Mather, L. Malmgard, C. Lewis, C. Asselin-Paturel, F. Brière, G. Trinchieri, and C. A. Brion.** 2002. Interferon α/β and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression *in vivo*. J. Exp. Med. **195:**517–528.
9. **Dalod, M., T. Hamilton, R. Salomon, T. P. Salazar-Mather, S. C. Henry, J. D. Hamilton, and C. A. Biron.** 2003. Dendritic cell responses to early cytomegalovirus infection: subset functional specialization and differential regulation by interferon α/β. J. Exp. Med. **197:**885–898.
10. **Diebold, S. S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L. E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow, and C. Reis e Sousa.** 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. Nature **424:**324–328.
11. **Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna.** 2006. Essential role of MDA5 in type I IFN responses to polyriboinosinic acid and encephalomyocarditis picornavirus. Proc. Natl. Acad. Sci. USA **103:**8459–8464.
12. **Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena.** 2002. Antigen presentation and T cell stimulation by dendritic cells. Annu. Rev. Immunol. **20:**621–676.
13. **Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740–745.
14. **Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira.** 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat. Immunol. **3:**196–200.
15. **Honda, K., H. Yanai, A. Takaoka, and T. Taniguchi.** 2005. Regulation of type I IFN induction: a current view. Int. Immunol. **17:**1367–1378.
16. **Hoshino, K., T. Kaisho, T. Iwabe, O. Takeuchi, and S. Akira.** 2002. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. Int. Immunol. **14:**1225–1231.
17. **Iwasaki, A., and R. Medzhitov.** 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. **5:**987–995.
18. **Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, O. Yamaguchi, K. Ohtsu, T. Tsujimura, C. S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira.** 2006. Differential role of MDA5 and RIG-I in the recognition of RNA viruses. Nature **141:**101–105.
19. **Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira.** 2005. Cell-type specific involvement of RIG-I in antiviral response. Immunity **23:**19–28.
20. **Kawai, T., and S. Akira.** 2006. Innate immune recognition of viral infection. Nat. Immunol. **7:**131–137.
21. **Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira.** 2005. IPS-1, an adaptor triggering RIG-I- and MDA5-mediated type I interferon induction. Nat. Immunol. **6:**1074–1076.
22. **Kolumam, G. A., T. Sunil, L. J. Thompson, J. Sprent, and K. Murali-Krishna.** 2005. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J. Exp. Med. **202:**637–650.
23. **Kumagai, Y., O. Takeuchi, H. Kato, H. Kumar, K. Matsui, E. Morii, K. Aozasa, T. Kawai, and S. Akira.** 2007. Alveolar macrophages are the primary interferon-α producer in pulmonary infection with RNA viruses. Immunity **27:**240–252.
24. **Kumar, H., T. Kawai, H. Kato, S. Sato, K. Takahashi, C. Coban, M. Yamamoto, S. Uematsu, K. J. Ishii, O. Takeuchi, and S. Akira.** 2006. Essential role of IPS-1 in innate immune responses against RNA viruses. J. Exp. Med. **203:**1795–1803.
25. **Le Bon, A., and D. F. Tough.** 2002. Links between innate and adaptive immunity via type-I interferon. Curr. Opin. Immunol. **14:**432–436.
26. **Matloubian, M., M. Suresh, A. Glass, M. Galvan, K. Chow, J. K. Whitmire, C. M. Walsh, W. R. Clark, and R. Ahmed.** 1999. A role for perforin in downregulating T cell responses during chronic viral infections. J. Virol. **73:**2527–2536.
27. **Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp.** 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature **437:**1167–1172.
28. **Meylan, E., J. Tschopp, and M. Karin.** 2006. Intracellular pattern recognition receptors in the host response. Nature **442:**39–44.
29. **Montoya, M., M. J. Edwards, D. M. Reid, and P. Borrow.** 2005. Rapid activation of spleen dendritic cell subsets following lymphocytic choriomeningitis virus infection of mice: analysis of the involvement of type I IFN. J. Immunol. **174:**1851–1861.
30. **Oldstone, M. B. A.** 2002. Biology and pathogenesis of lymphocytic choriomeningitis virus infection. Curr. Top. Microbiol. Immunol. **263:**83–117.
31. **Otheki, T., M. Parsons, A. Zakarian, R. G. Jones, L. T. Nguyen, J. R. Woodgett, and P. S. Ohashi.** 2000. Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. J. Exp. Med. **192:**99–104.
32. **Ou, R., S. Zhou, L. Huang, and D. Moskophidis.** 2001. Critical role for alpha/beta and gamma interferons in persistence of lymphocytic choriomeningitis virus by clonal exhaustion of cytotoxic T cells. J. Virol. **75:**8407–8423.
33. **Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen.** 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF3. Cell **122:**669–682.
34. **Stetson, D. B., and R. Medzhitov.** 2006. Type I interferons in host defense. Immunity **25:**373–381.
35. **Sun, Q., L. Sun, H. H. Liu, X. Chen, R. B. Seth, J. Forman, and Z. J. Chen.** 2006. The specific and essential role of MAVS in antiviral innate immune responses. Immunity **24:**633–642.
36. **Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira.** 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity **11:**443–451.
37. **Uematsu, S., M. H. Jang, N. Chevrier, Z. Guo, Y. Kumagai, M. Yamamoto, H. Kato, N. Sougawa, H. Matsui, H. Kuwata, H. Hemmi, C. Coban, T. Kawai, K. Ishii, O. Takeuchi, M. Miyasaka, K. Takeda, and S. Akira.** 2006. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c$^+$ lamina propria cells. Nat. Immunol. **7:**868–874.
38. **Uematsu, S., S. Sato, M. Yamamoto, T. Hirotani, H. Kato, F. Takeshita, M. Matsuda, C. Coban, K. Ishii, T. Kawai, O. Takeuchi, and S. Akira.** 2005. Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-α induction. J. Exp. Med. **201:**915–923.
39. **Xu, L. G., Y. Y. Wang, K. J. Han, L. Y. Li, Z. Zhai, and H. B. Shu.** 2005. VISA is an adaptor protein required for virus-triggered IFN-β signaling. Mol. Cell **19:**727–740.
40. **Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira.** 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. Science **301:**640–643.
41. **Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K.**

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

Taira, E. Foy, Y. M. Loo, M. Gale, S. Akira, S. Yonehara, A. Kato, and T. Fujita. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. **175:** 2851–2858.

42. **Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. **5:**730–737.

43. **Zhou, S., E. A. Kurt-Jones, L. Mandell, A. Cerny, M. Chan, D. T. Golenbock, and R. W. Finberg.** 2005. MyD88 is critical for the development of innate and adaptive immunity during acute lymphocytic choriomeningitis virus infections. Eur. J. Immunol. **35:**822–830.

Downloaded from jvi.asm.org at OSAKA UNIV BIKEN MI on February 18, 2008

Osamu Takeuchi
Shizuo Akira

Recognition of viruses by innate immunity

Authors' address
Osamu Takeuchi, Shizuo Akira
Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, ERATO, Japan Science and Technology Agency, Osaka, Japan.

Correspondence to:
Shizuo Akira
Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, ERATO, Japan Science and Technology Agency, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan
Tel: +81 6 6879 8303
Fax: +81 6 6879 8305
e-mail: sakira@biken.osaka-u.ac.jp

Acknowledgements
We thank M. Hashimoto for secretarial assistance. This work was supported in part by grants from the Special Coordination Funds of the Japanese Ministry of Education, Culture, Sports, Science and Technology, the 21st Century Center of Excellence Program of Japan, and the NIH (P01 AI070167).

Summary: The innate immune system plays critical roles in recognizing viral infections and evoking initial anti-viral responses. Nucleotides from RNA viruses are recognized by retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and Toll-like receptors (TLRs), and the recognition triggers signaling cascades that induce anti-viral mediators such as type I interferons (IFNs) and pro-inflammatory cytokines. The RLH signaling pathways play essential roles in the recognition of RNA viruses in various cells, with the exception of plasmacytoid dendritic cells (pDCs). However, TLRs are important for the production of type I IFNs in pDCs but not in other cell types. The contributions of RLHs and TLRs to the production of type I IFNs in response to RNA viruses vary depending on the route of infection. Specifically, local infections induce IFNs through RLHs but not TLRs, whereas systemic infections strongly stimulate TLRs in pDCs. In this review, we discuss recent advances toward clarifying the signaling pathways activated by RLHs and TLRs.

Keywords: *type I interferon, Toll-like receptor, RIG-I-like helicase, RNA virus*

Introduction

Viruses that have invaded a host are initially recognized by infected non-immune cells as well as innate immune cells such as macrophages and dendritic cells (DCs), which rapidly evoke anti-viral responses via the production of type I interferons (IFNs) and pro-inflammatory cytokines. Type I IFNs, comprised of multiple IFN-α isoforms and a single IFN-β, and other members, such as IFN-ω, -ε, and -κ, are pleiotropic cytokines that are essential for anti-viral immune responses (1, 2). They induce apoptosis of virus-infected cells and cellular resistance to viral infection, in addition to activating natural killer (NK) and T cells. Thus, type I IFNs play essential roles not only in innate anti-viral responses but also in activating the adaptive immune system.

The expressions of type I IFN and cytokine genes are regulated by intracellular signaling cascades that are activated by germline-encoded pattern recognition receptors. These receptors recognize molecular patterns specific to microorganisms, such as bacterial lipopolysaccharide, bacterial lipoproteins, microbial proteins, and nucleotides. Three classes of innate pattern-recognition receptors have

Immunological Reviews 2007
Vol. 220: 214–224
Printed in Singapore. *All rights reserved*

© 2007 The Authors
Journal compilation © 2007 Blackwell Munksgaard
Immunological Reviews
0105-2896

been identified, namely Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins.

TLRs are comprised of leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic domain designated the Toll/interleukin-1 receptor (IL-1R) homology (TIR) domain (3, 4). To date, 10 and 12 TLRs have been reported in humans and mice, respectively, and the microbial components recognized by each TLR have mostly been identified. TLR3, TLR7, and TLR9 are localized on cytoplasmic vesicles, such as endosomes and endoplasmic reticulum, and recognize microbial nucleotides. Specifically, TLR3 detects double-stranded (ds) RNA, while TLR7 and TLR9 recognize single-stranded (ss) RNA and DNA with a CpG motif, respectively. The TIR domain of TLRs is localized in the cytoplasm, and the LRRs face the extracellular space or the endosomal lumen. Because the LRRs but not the TIR domain are responsible for microbial sensing, TLRs are not suitable for recognizing viruses that have infected cells and are localized in the cytoplasm or the nucleus.

Viruses that have invaded a cell are recognized by receptors localized in the cytoplasm. RIG-I and its paralogues, collectively referred to as RLHs, are responsible for the recognition of RNA molecules derived from RNA viruses in a cell. RLH-mediated signaling triggered by RNA virus infection induces the expressions of type I IFN genes as well as pro-inflammatory cytokine genes. A member of the NLR family has also been implicated in the recognition of viral RNA in the cytoplasm. In this review, we focus on the roles of RLHs in RNA virus recognition, followed by the roles of the TLR system. Finally, we discuss the relationship between these two virus recognition mechanisms during the course of RNA virus infection *in vivo*.

Recognition of RNA viruses by RLHs

Viruses replicate in infected cells by utilizing the host's machineries. dsRNAs are present in cells as intermediates of viral RNA replication, dsRNA virus genomes, or stem-loop RNAs encoded by some DNA viruses. RIG-I was originally cloned as a cytoplasmic protein that mediates dsRNA-induced type I IFN production. RIG-I is comprised of two N-terminal caspase recruitment domains (CARDs), followed by a DExD/H box helicase domain (5). RIG-I forms a family with melanoma differentiation-associated gene-5 (MDA5) (also known as Helicard) and LGP2, based on the high similarities of their helicase domains (6–8). The expressions of these proteins are strongly induced by IFNs, and they interact with dsRNAs through their helicase domains. Overexpression of RIG-I CARDs alone induces activation of the IFN-β promoter, indicating that CARDs are responsible for triggering downstream signaling cascades. However, the adenosine triphosphatase (ATPase) activity of the helicase domain has been shown to be critical for the recognition of dsRNAs by RIG-I. Besides the CARDs and the helicase domain, a C-terminal portion of RIG-I, designated the repressor domain (RD), was found to be important for controlling RIG-I-mediated IFN responses (9). Briefly, RIG-I without the RD constitutively activated the IFN-β promoter, while overexpression of the RD inhibited RIG-I-IPS-1 binding as well as virus-induced IFN-β promoter activation.

The functional roles of RIG-I and MDA5 *in vivo* have been investigated by generating mice deficient in these molecules (10–12). Although MDA5 $^{-/-}$ mice did not show any developmental abnormalities, RIG-I $^{-/-}$ mice mostly showed embryonic lethality, on the 129Sv × C57BL/6 background, due to fetal liver apoptosis. However, RIG-I $^{-/-}$ mice overcame this developmental defect on the CD1 background. Further analyses of RIG-I $^{-/-}$ and MDA5 $^{-/-}$ mice revealed that RIG-I and MDA5 recognize different RNA viruses. RIG-I $^{-/-}$ fibroblasts and conventional DCs (cDCs) did not produce type I IFNs in response to various RNA viruses, including paramyxoviruses, influenza virus, vesicular stomatitis virus (VSV), and Japanese encephalitis virus (JEV) (Fig. 1). Although MDA5 $^{-/-}$ cells exhibited normal production of type I IFNs in response to the viruses recognized by RIG-I, they failed to respond to encephalomyocarditis virus (EMCV), Theiler's virus, and Mengo virus. These latter viruses belong to the picornavirus family, indicating that MDA5 specifically recognizes picornavirus infections. Consistently, RIG-I $^{-/-}$ and MDA5 $^{-/-}$ mice were highly susceptible to VSV and EMCV, respectively. Furthermore, EMCV infection failed to induce the production of type I IFNs and pro-inflammatory cytokines in MDA5 $^{-/-}$ mice. These observations indicate that RLH-mediated recognition of RNA viruses plays critical roles in the host defenses against viral infection. In humans, a non-synonymous single nucleotide polymorphism (SNP) in MDA5 was reported to show an association with type I diabetes (13). Specifically, the A946T substitution in MDA5 was more frequently identified in type I diabetes patients. Although it is not clear whether this SNP affects the IFN-inducing activity of MDA5, the finding is interesting because the cause of type I diabetes is strongly connected to viral infection.

Similar to the case for viruses, synthetic RNAs are also differentially recognized by RIG-I and MDA5. MDA5 was

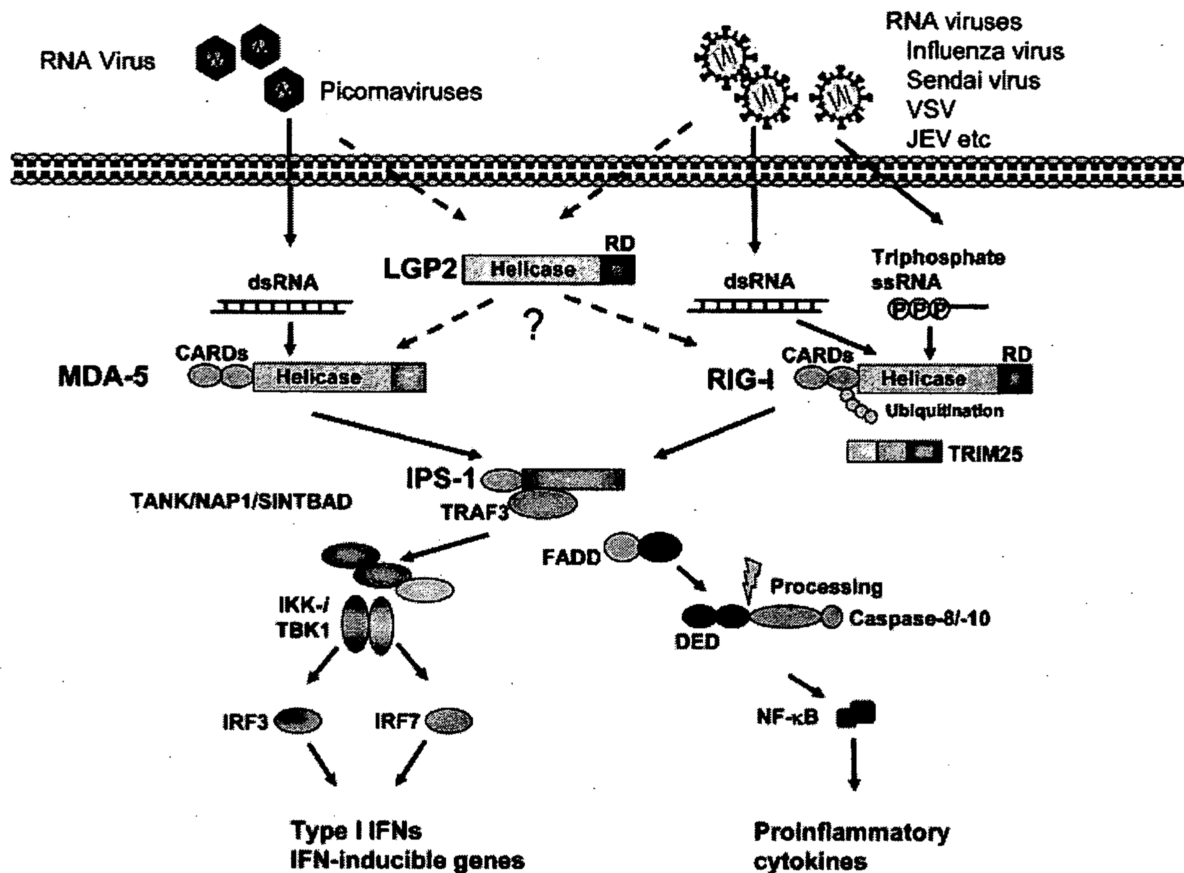

Fig. 1. RLH- and TLR3-mediated viral detection. RIG-I and MDA5 recognize dsRNA and 5′-triphosphate ssRNA from RNA viruses such as influenza virus, Sendai virus, VSV, JEV, and picornaviruses. RIG-I and MDA5 trigger signaling cascades via IPS-1, a CARD-containing protein. IPS-1 induces type I IFN gene expression via TRAF3, FADD, TANK, NAP1, SINTBAD, TBK1/IKK-i, and IRF-3/IRF-7. NF-κB is also activated by IPS-1 via a FADD and caspase-8/-10-dependent pathway through their cleavage. TRIM25-mediated ubiquitination of RIG-I facilitates the activation of RIG-I signaling. The role of LGP2 in RNA virus recognition is still unclear. TLR, Toll-like receptor; IFN, interferon; RLH, retinoic acid-inducible gene I (RIG-I)-like helicases; VSV, vesicular stomatitis virus; JEV, Japanese encephalitis virus; FADD, FAS-associated death domain-containing protein; TANK, TRAF-associated NF-κB activator; TRAF3, tumor necrosis factor-receptor associated factor 3; NAP1, NAK-associated protein 1; TRIM25, tripartite motif 25; SINTBAD, similar to NAP1 TBK1 adapter; IRF-3, IFN-regulatory factor-3; CARD, caspase recruitment domains; IPS, IFN-β promoter stimulator.

found to recognize polyinosinic polycytidylic acid [poly(I:C)], because MDA5$^{-/-}$ cDCs failed to produce type I IFNs in response to poly(I:C) and MDA5$^{-/-}$ mice showed severely impaired production of type I IFNs in response to poly(I:C) administration (11). In contrast, RIG-I detects in vitro-transcribed dsRNAs. RNAs synthesized by T7 polymerase *in vitro* are known to contain 5′-triphosphate. Recently, 5′-triphosphate ssRNA was shown to stimulate IFN production by RIG-I but not MDA5 (14, 15). Thus, RIG-I can discriminate between 5′-triphosphorylated RNAs from some viruses and host mRNAs whose 5′ ends are capped.

Cells infected with EMCV but not influenza virus generate dsRNA. Furthermore, the 5′-end of the EMCV genomic RNA is covalently linked to a peptide called VPg, such that 5′-phosphate is not present in EMCV. Dephosphorylation of 5′-triphosphate RNA or the influenza genome results in a loss of their ability to induce IFNs, suggesting that recognition by RIG-I is mediated through 5′-triphosphate ssRNA. However, small dsRNAs (ranging from 21 to 27 nucleotides) without 3′ overhangs have been reported to induce IFN-inducible genes via RIG-I (16). Small dsRNAs with 3′ overhangs of two nucleotides, which can be the product of Dicer-mediated processing, fail to induce RIG-I helicase activity. Thus, it is still unclear whether the helicase activity of RIG-I is required for the recognition of RNAs and RNA viruses. In contrast, the RNA structure responsible for MDA5 recognition has not been identified.

RIG-I is able to recognize not only RNA viruses but also the Epstein–Barr virus (EBV), which belongs to the herpesvirus family (17). EBV-encoded small RNAs were reported to be responsible for RIG-I-mediated IFN-β production. However, another DNA virus, vaccinia virus, activated type I IFN

responses independent of the presence of RIG-I and/or MDA5 (18). Thus, the roles of RLHs in the recognition of DNA viruses need to be explored further.

Several reports have shown recently that RIG-I-mediated signaling is controlled by ubiquitination of RIG-I. First, the CARDs of RIG-I undergo Lys 63-linked ubiquitination by tripartite motif 25 (TRIM25), a ubiquitin E3 ligase composed of a RING finger domain, a B-box/coiled-coil domain, and an SPRY domain (19). This ubiquitination is necessary for efficient activation of the RIG-I signaling pathway, and TRIM25 $^{-/-}$ cells display impaired production of type I IFNs against viral infection. RIG-I also undergoes ubiquitination by the ubiquitin ligase RNF125, which leads to its proteasomal degradation (20). Thus, RIG-1 ubiquitination by RNF125 is considered to inhibit aberrant activation of RIG-I signaling.

Because LGP2 lacks a CARD, it is suggested to function as a negative regulator of RIG-I/MDA-5 signaling (6, 21). Overexpression of LGP2 inhibited Sendai virus and Newcastle disease virus (NDV) signaling. LGP2 was reported to bind to dsRNA, thereby preventing RIG-I-mediated recognition. LGP2 also contains an RD, similar to the case for RIG-I (9). The LGP2 RD was found to interact with the RD of RIG-I and to suppress the self-association of RIG-I. Recently, Lgp2 $^{-/-}$ mice were generated and analyzed by Barber and colleagues (22). Their Lgp2 $^{-/-}$ mice showed highly elevated induction of type I IFNs in response to poly(I:C) stimulation. The production of IFN-β in response to VSV infection was modestly increased in Lgp2 $^{-/-}$ MEFs, and Lgp2 $^{-/-}$ mice were more resistant to VSV infection than control mice. However, Lgp2 $^{-/-}$ mice showed partially impaired type I IFN production in response to EMCV infection. Barber and colleagues (22) proposed that LGP2 is a negative regulator of RIG-I but not of MDA5; however, given that both poly(I:C) and EMCV are recognized by MDA5, the difference cannot be explained by differential usage of LGP2 for RIG-I and MDA5 signaling. However, further studies are required for clarifying the mechanisms of LGP2-mediated RNA virus recognition.

RLH signaling pathways

The CARDs of RIG-I and MDA5 are responsible for initiating signaling cascades. RIG-I and MDA5 associate with an adapter protein, IFN-β promoter stimulator-1 (IPS-1), also known as MAVS, VISA, or CARDIF, which contains an N-terminal CARD (23–26) (Fig. 1). The CARD of IPS-1 interacts with the CARDs of RIG-I and MDA5. Overexpression of IPS-1 induces the activation of IFN-β promoters as well as a transcription factor NF-κB. IPS-1 is localized on the outer mitochondrial membrane through a C-terminal transmembrane domain. IPS-1 $^{-/-}$ mice were found to be defective in the production of type I IFNs and pro-inflammatory cytokines in response to all RNA viruses recognized by either RIG-I or MDA5 (27, 28). Interestingly, IPS-1 $^{-/-}$ mice did not show any developmental defects. IPS-1 $^{-/-}$ mice were highly susceptible to infection with VSV and EMCV, the viruses recognized by RIG-I and MDA5. These findings indicate that IPS-1 plays an essential role in RIG-I/MDA5 signaling. Downstream of IPS-1, tumor necrosis factor (TNF)-receptor-associated factor 3 (TRAF3) has been shown to be important for the production of IFNs (29, 30). The C-terminal TRAF domain of TRAF3 associates with a TRAF-interaction motif found in the proline-rich region of IPS-1 (31). TRAF3 $^{-/-}$ cells showed impaired production of type I IFNs in response to viral infection.

Two inhibitors of NF-κB (IκB) kinase (IKK)-related kinases, namely TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and inducible IκB kinase [(IKK-i) also known as IKKε], are activated in the downstream of TRAF3 (32–36). These kinases phosphorylate IFN-regulatory factor-3 (IRF-3) and IRF-7. The activated IRF-3 and IRF-7 subsequently translocate into the nucleus where they bind to IFN-stimulated response elements (ISREs), resulting in the expressions of type I IFN and IFN-inducible genes (37). Although mice lacking TBK1 and IKK-i showed embryonic lethality, cDCs prepared from the fetal liver failed to respond to various viruses (35, 38). Secreted type I IFNs activate cells in an autocrine or paracrine manner via Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways, thereby leading to the expression of a set of IFN-inducible genes (1, 2). Recently, IKK-i was also reported to function as a kinase that phosphorylates STAT1 and controls a subset of IFN-inducible genes (39). Cells lacking both IRF-3 and IRF-7 did not produce type I IFNs in response to viral infection. Nevertheless, IRF-3 $^{-/-}$ IRF-7 $^{-/-}$ mice did not show embryonic lethality, indicating that TBK1 and IKK-i control not only IRFs and STAT1 but also other signaling pathways. Indeed, *Drosophila* IKK-i was reported to promote the degradation of *Drosophila* inhibitor of apoptosis protein 1 (IAP1) by phosphorylation and to be required for proper sensory organ precursor development through its control of caspase-independent cell death (40). Thus, further studies are required to assess the roles of TBK1 and IKK-i in mammals.

TBK1 and IKK-i interact with TANK (41, 42). TANK was reported to form a complex with IPS-1, TRAF3, and TBK1/IKK-i, and knockdown of TANK resulted in reduced type I IFN production (43). NAK-associated protein 1 (NAP1), a protein homologous to TANK, is also involved in RLH-mediated

responses (44). Recently, another protein showing homology with TANK, named SINTBAD (similar to NAP1 TBK1 adapter), was also reported to activate TBK1 and IKK-i in RLH signaling (45). It is not clear whether TANK, NAP1, and SINTBAD function redundantly or have distinct roles in macrophages and DCs. Further studies are required for assessing the role of these molecules *in vivo*.

FAS-associated death domain-containing protein (FADD) has been reported to be required for type I IFN production in response to dsRNA as well as viral infection, although another study showed that virus-induced type I IFN responses were not impaired in FADD$^{-/-}$ cells (6, 46). Furthermore, FADD was identified as a protein that interacts with caspase-8/-10 by yeast two-hybrid screening. Coimmunoprecipitation experiments revealed that FADD was able to interact with IPS-1. IPS-1 activation led to cleavage of caspase-8/-10, and the freed death effecter domains activated NF-κB, thereby controlling IPS-1-dependent pro-inflammatory cytokine expression (47).

In summary, RIG-I and MDA5 play critical roles in the recognition of RNA viruses in cDCs, macrophages, and fibroblasts. They trigger common signaling cascades that lead to the production of type I IFNs and pro-inflammatory cytokines.

TLR3-dependent signaling pathway

In addition to RLHs, TLR3 is also able to recognize dsRNA and poly(I:C) in macrophages. TLR3 activates the IFN-β promoter as well as NF-κB in response to poly(I:C) stimulation. TLR3$^{-/-}$ macrophages showed impaired IFN-β gene expression and production of IL-6 and IL-12 in response to poly(I:C) addition to culture media (11, 48). However, the role of TLR3 *in vivo* is still controversial. When poly(I:C) was administered intravenously, TLR3$^{-/-}$ mice showed normal production of IFN-α and IFN-β in their sera, whereas the production of IL-12p40 was severely impaired (48). These results indicate that poly(I:C)-induced production of type I IFNs *in vivo* is mainly mediated by MDA5 but not by TLR3. TLR3 is responsible for controlling pro-inflammatory cytokines, rather than type I IFNs, in response to poly(I:C). TLR3$^{-/-}$ mice showed severely impaired cytotoxic T lymphocyte (CTL) cross-priming against poly(I:C)-loaded virally infected cells (49). TLR3 signaling was also reported to be partly required for poly(I:C)-induced anti-tumor NK cell activation (50).

TLR3$^{-/-}$ mice were found to be more susceptible to mouse cytomegalovirus infection (MCMV) than wildtype mice. Nevertheless, TLR3$^{-/-}$ mice were more resistant to infection with several ssRNA viruses, such as West Nile virus, Phlebovirus, and influenza virus (51–53). TLR3$^{-/-}$ mice showed diminished virus titers in the brain, together with reduced cytokine production in the periphery in response to West Nile virus infection. Furthermore, the presence of TLR3 was required for peripheral West Nile virus to be able to break through the blood–brain barrier and establish brain infection (51). In response to Phlebovirus or influenza virus infection, TLR3$^{-/-}$ mice exhibited diminished pro-inflammatory responses, suggesting that cytokines and chemokines produced in wildtype mice were deleterious to the survival of the mice (52, 53). These observations suggest that these viruses take advantage of TLR3-mediated inflammatory responses for establishing infection.

Upon stimulation with poly(I:C), TLR3 triggers a signaling cascade via a TIR domain-containing adapter inducing IFN-β (TRIF) (also known as TICAM-1) (54, 55). TRIF$^{-/-}$ macrophages showed impaired induction of genes encoding IFN-β as well as IFN-inducible genes in response to poly(I:C) stimulation (56). TRIF was also responsible for IFN-β production in response to bacterial lipopolysaccharide, the TLR4 ligand (56, 57). TRIF associates with TRAF3 and TRAF6 through TRAF-binding motifs present in its N-terminal portion (29, 30, 58). TRIF also contains a C-terminal receptor-interacting protein (RIP) homotypic interaction motif (RHIM), and TRIF interacts with RIP1 and RIP3 via the RHIM (59). TRAF6 and RIP1 are able to activate NF-κB. In contrast, TRAF3 is responsible for inducing type I IFNs (29, 30). TANK and NAP1 are also involved in TRIF-mediated type I IFN production. TBK1 and IKK-i are responsible for the activation of a TRIF-dependent pathway, indicating that the signaling pathways triggered by TLR stimulation and RIG-I converge at the level of TRAF3-TBK1/IKK-i.

In addition to the TRIF-dependent signaling pathway, TLR3 was reported to activate the phosphatidylinositol-3 kinase (PI3K) pathway (60). Specifically, tyrosine phosphorylation of TLR3 induced PI3K recruitment to the receptor, and subsequent activation of Akt led to full phosphorylation and activation of IRF-3.

TLR3 plays important roles in evoking inflammatory responses, rather than type I IFN production, against RNA virus infection *in vivo*. Thus, TLR3 signaling could be beneficial or deleterious depending on the type of virus infection.

Recognition of RNA viruses in plasmacytoid DCs

Plasmacytoid DCs (pDCs) are known to produce high levels of type I IFNs in response to viral infection (61). pDCs express