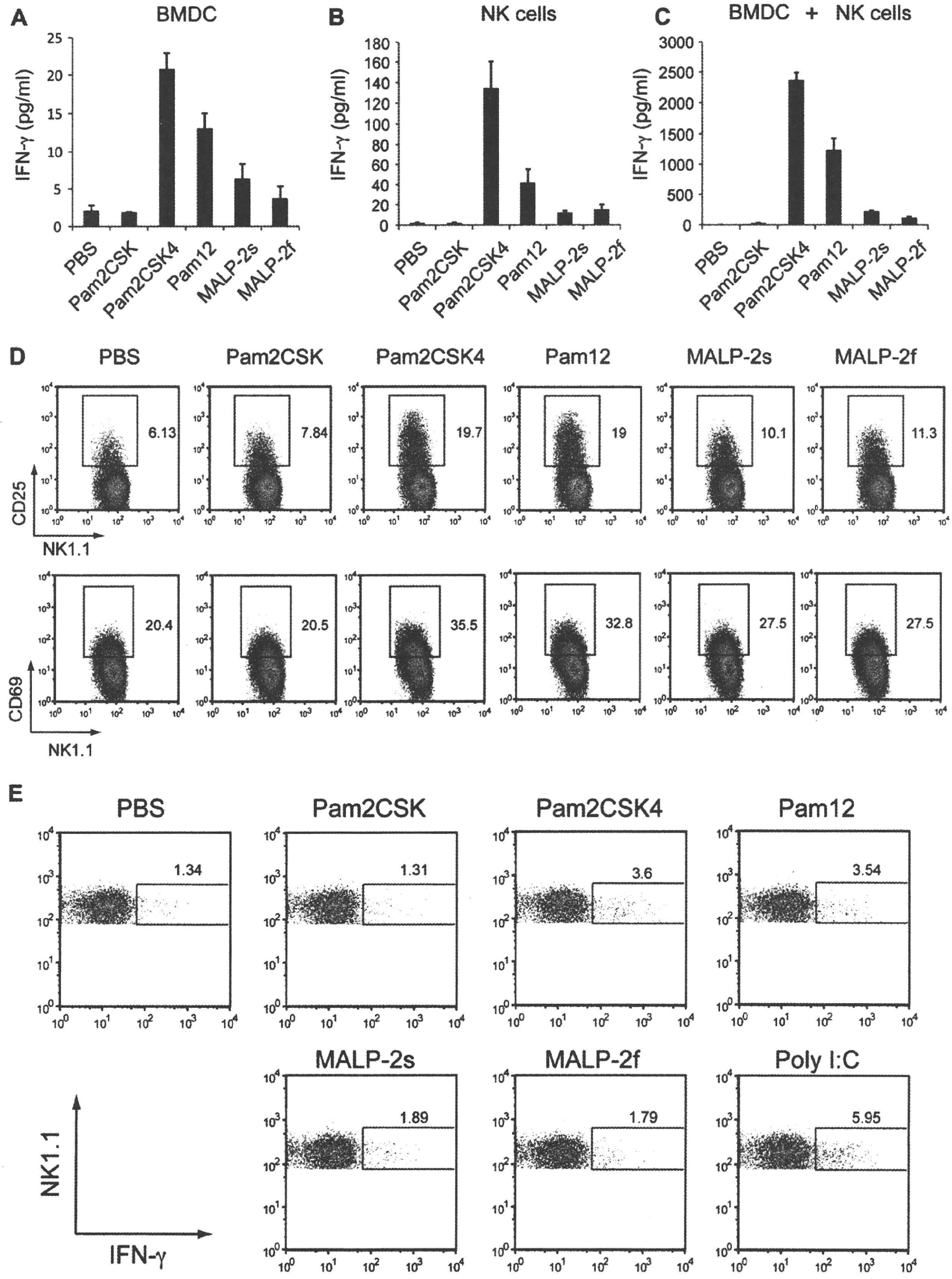

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The degrees of CD86 up-regulation were examined with these lipopeptides, and similar DC maturation was evaluated by flow cytometry (Fig. 1D,E). Although the levels of CD86 were increased in response to Pam2 peptides (100 nM), no significant difference was observed among the Pam2 peptides tested (Fig. 1E). At the dose of Pam2 where cytokine induction sufficiently occurs, CD86 expression inadequately takes place in BMDC, as reported previously [12]. Thus, NF-κB activation, cytokine liberation and DC maturation are partially co-related in these lipopeptides.

3.2. NK activation by Pam2CSK4 and MALP-2

Previous reports suggested that TLR2 agonists have ability to induce NK activation [11,12]. To investigate whether the *Mycoplasma* lipopeptides harbor activity of NK cell activation, we added the Pam2Cys peptides (100 nM) to BMDC, NK cells or BMDC/NK co-culture as in a previous method assessing polyI:C activity for BMDC-mediated NK activation [10]. Although BMDC *per se* can induce IFN-γ production in response to some TLR stimuli [17,18], we could detect only minute amounts of IFN-γ in our setting using Pam2 lipopeptides (Fig. 2A). In this context, three markers for NK activation [19] were assessed with this system, IFN-γ production, up-regulation of NK activation markers and target cell (B16D8) cytotoxicity by NK cells (Fig. 2). IFN-γ was generated in the supernatants (sup) of NK cells (Fig. 2B) or BMDC/NK co-culture (Figs. 2C and 3A left hand panel) in response to the control lipopeptides, Pam2CSK4 and Pam2-Cys12. However, MALP-2s and f showed significantly low potentials for IFN-γ-induction comparable to the negative control Pam2CSK.

The NK cell activation markers CD25 and CD69 were analyzed with BMDC co-cultured NK cells with or without the lipopeptide treatment by flow cytometry (Fig. 2D). Up-regulation of surface CD25 and CD69 was observed in NK cells incubated with BMDC stimulated with Pam2CSK4 and Pam2Cys12 but far less with MALP-2s and f.

Activated NK cells are a major source of IFN-γ which causes a variety of responses of the immune system. To further examine whether BMDC matured with Pam2CSK4 or MALP-2 drive NK-dependent IFN-γ secretion, we stimulated BMDC with these lipopeptide reagents for 4 h and then mixed with NK cells (ratio 1:2) for 20 h. Breferdin was added to the mixture in order to accumulate IFN-γ in the NK cells for the last 4 h of incubation. As shown in Fig. 2E, the TLR2 ligands Pam2CSK4 and Pam2Cys12 significantly increased the frequency of IFN-γ-secreting NK cells, while MALP-2f and s showed far less activity to produce IFN-γ in the NK cells.

Cytotoxic activity was evaluated using B16D8 cells as a target [10]. BMDC-activated NK cells (see above) were incubated with B16D8 cells at a ratio of 30:1. Again, MALP-2s and f showed less effective killing against the target (Fig. 3B left hand panel). The other lipopeptides had sufficient killing activity compared to the control polyI:C: one of two examples assayed with different BMDC lots are shown in the figure.

3.3. Participation of TLR2/MyD88 in MALP-2-mediated NK activation

We next examined whether the lipopeptide-mediated IFN-γ secretion was dependent on MyD88 of BMDC. IFN-γ secretion was almost completely abrogated in the co-culture with MyD88−/− BMDC and wild-type NK cells in the presence of Pam2Cys12 and Pam2CSK4 (Fig. 3A), Similar tendencies were observed with MALP-2 peptides, which essentially evoked a minimal IFN-γ production, and the IFN-γ-induction was largely abrogated with MyD88−/− BMDC (Fig. 3A). The results were less prominently reproduced with TLR2−/− BMDC and wild-type NK cells (data not shown). Further confirmation was performed using the mixtures with wild-type NK cells and various lipopeptides. No direct activation of NK cells was observed in the absence of BMDC (data not shown).

The results were further confirmed with NK cytotoxic assay using NK cells co-cultured with Pam2Cys lipopeptide-stimulated BMDC (Fig. 3B). When wild-type BMDC was used as an NK cell cytotoxicity inducer, full NK activation was induced by Pam2Cys12 or Pam2CSK4. MALP-2f and s were found to be inefficient NK activators (Fig. 3B). If wild-type BMDC were replaced with MyD88−/− BMDC, BMDC-enhanced NK cytotoxicity was abrogated (Fig. 3B). The MyD88 pathway in BMDC is crucial for BMDC-mediated NK cell activation.

3.4. Combinational recognition of MALP-2s and f by TLR2 and TLR6

TLR2 recognizes diacyl lipopeptide in combination with TLR6 [20] while TLR2 recognizes triacyl lipopeptide together with TLR1 [21]. We found TLR2/6 cooperate to recognize *Staphylococcus aureus* lipopeptides using HEK293 cells with TLR2/6 expression. Data testing MALP-2f for the usage of TLR2/6 are shown in Fig. 4. Single receptors of TLR1, TLR6 and TLR10 barely activate NF-κB by reporter assay and only TLR2 exhibited <60 fold ELAM promoter activation (data not shown). No enhanced activation was observed in combination with TLR2 and TLR1 or TLR2 and TLR10. Similar results on TLR2/6-mediated augmentation of reporter activation were observed with MALP-2s and Pam2CSK4 (data not shown).

Fig. 2. Pam2Cys-containing peptides activate NK cells through TLR2 in BMDC. (A, B, C) BMDC and NK cells prepared from wild-type mice were stimulated with Pam2Cys-containing peptides for 24 h (A, B). Alternatively, BMDC were stimulated with Pam2Cys-containing peptides for 4 h. Then, NK cells prepared from wild-type mouse spleen were co-cultured with the BMDC for 24 h. IFN-γ levels in the culture supernatant are shown. (D) CD25 and CD69 expression on NK cells co-cultured with BMDC in the presence of Pam2Cys-containing peptides. The NK cell populations (marked with NK1.1) were gated on the display of FACS and levels of CD25 or CD69 (inset values) were examined as shown in the graphs. (E) Intracellular IFN-γ staining of NK cells co-cultured with BMDC in the presence of Pam2Cys-containing peptides as above. Cells were treated with breferdin and then permeabilized. Intracellular IFN-γ was detected by specific mAb. IFN-γ positive cells are marked with square and their frequencies are indicated by inset values.

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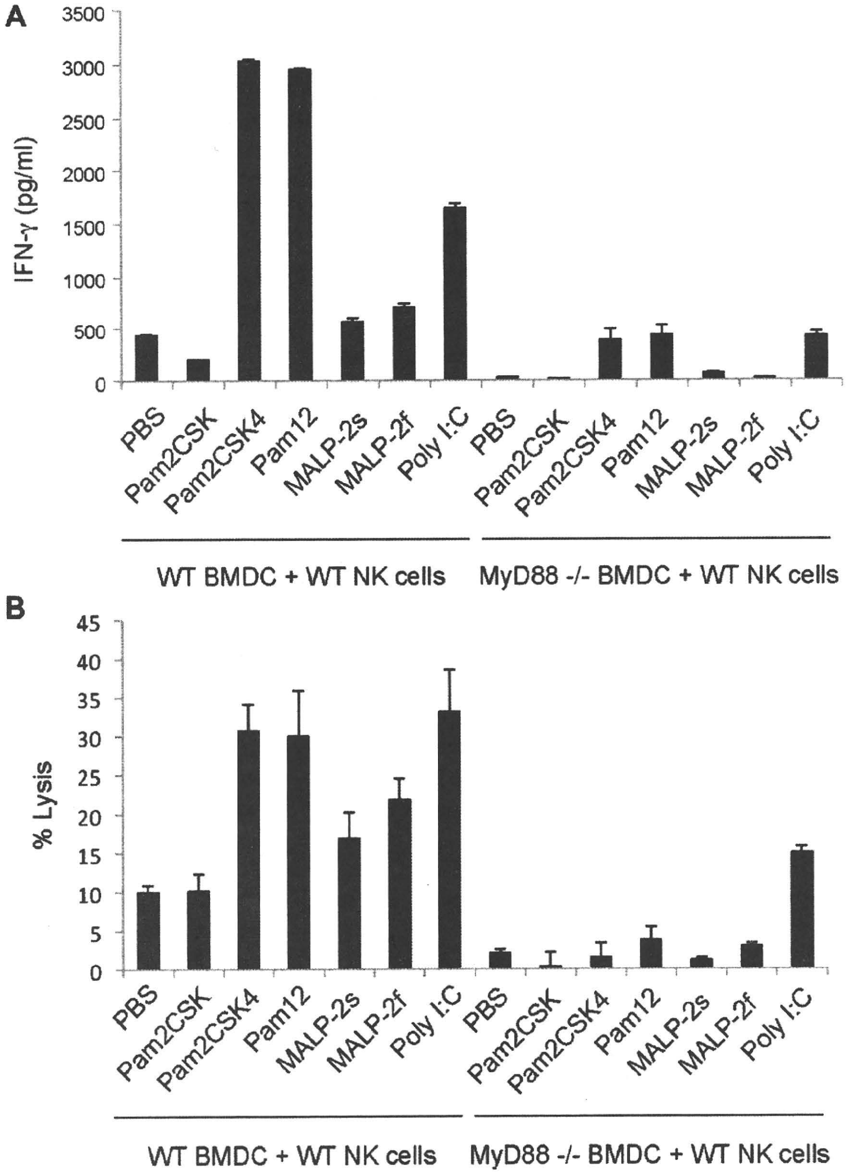

Fig. 3. IFN-γ production and cytotoxic activity of NK cells co-cultured with BMDC in the presence of Pam2Cys-containing peptides. Wild-type and MyD88−/− BMDC were stimulated with Pam2Cys-containing peptides for 4 h. Then, the BMDC were co-cultured with wild-type NK cells for 24 h (A) IFN-γ levels in culture supernatant were determined by ELISA. (B) Cytotoxic activities of NK cells were measured by ^{51}Cr release assay. B16D8 cells were used as a target. E/T ratio = 20.

Hence, TLR6 helps to amplify the TLR2 signal by MALP-2 lipopeptide as in other Pam2Cys lipopeptides, but IFN-γ was minimally induced in the NK cells.

3.5. *Antitumor adjuvant activity against NK-sensitive tumor* in vivo

Recent studies revealed that intratumoral or i.p. injection of MALP-2 supresses pancreatic carcinoma in a mouse model [22]. Tumor suppression is also observed with Pam2Cys type lipopeptides in B16D8 (NK-sensitive) implant mice [23]. The antitumor function by MALP-2 is abrogated in MyD88−/− mice, suggesting that TLR2/MyD88 and following cell-mediated immunity play a major part of tumor suppression [23]. We tested whether MALP-2 injected s.c. induces growth retardation of the tumor (NK target B16D8 cells) via NK activation (Fig. 5). Pam2CSK4 s.c. injected around tumor exhibited tumor growth retardation (Fig. 5A). This Pam2CSK4 activity was abrogated by injection of asialoGM-1 Ab (data not shown). In contrast, no tumor growth retardation was observed in this NK-sensitive tumor by s.c. injected MALP-2 (Fig. 5B). The results infer that MALP-2 exerts only minimal

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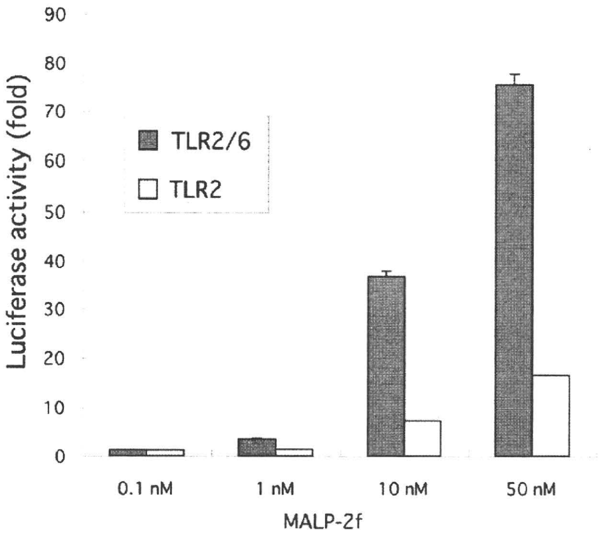

Fig. 4. TLR6 facilitates the recognition of MALP-2 by TLR2. HEK293 cells were transfected with the plasmids encoding TLR2 and/or TLR6, and ELAM-luciferase reporter. After 24 h, the cells were treated with MALP-2f for 6 h. Then, luciferase activity of the cell lysates was measured. Similar results were obtained with MALP-2s (not shown).

potential if any, to activate NK cells through BMDC *in vitro* and *vivo*. Unlike BCG-CWS [19] or polyI:C [8–10], MALP-2 barely suppresses tumor growth in this mouse system.

4. Discussion

Recent studies demonstrated that mDC induce NK activation by stimulation with TLR2 in mDC [12]. This NK activation occurs in a distinct mode of those reported in the TICAM-1/IPS-1 pathways for type I IFN induction because this mode of NK activation is derived through the MyD88 pathway in the mouse system. Indeed, MyD88 has been reported to participate in NK cell activation induced by *Plasmodium falciparum*-infected erythrocytes, but TLR2 response was not mandatory in the reported human case [24]. In another report, direct TLR2 stimulation in NK cells but not mDC was critical for NK cell activation in a vaccinia infection system [25]. We then tested whether a TLR2 agonist MALP-2 harbors adjuvant potential of mDC-mediated NK activation.

NK activation fails to be induced by MALP-2-stimulated mDC judged by IFN-γ production, up-regulation of NK activation marker CD25 and CD69, and cytotoxicity against the NK target B16D8 cells. Cytokines with NK activation properties such as IFN-α/β, IL-15 and IL-12p70 are not up-regulated in mDC in response to MALP-2, although a regulatory cytokine IL-10 is produced by stimulation with MALP-2 [3,5]. Finally, s.c. administration of MALP-2 did not result in retardation of tumor growth in mice with B16D8 tumor burden. Although Pam2CSK4 having 6 a.a.-stretch following the diacyl residue acts as an NK-activating reagent [10,18], two forms of MALP-2 with short (6 a.a.) or long (14 a.a.) peptide barely exhibits antitumor activity. Hence, NK activation is a phenotype induced by a limited group of Pam2Cys lipopeptides, and the peptide sequence is critical for inducing mDC-mediated NK activation. Our results infer that TLR2-dependent mDC response to drive NK activation largely relies on the peptide sequences of lipoproteins. TLR2-stimulating lipopeptides are not unifunctional: some are active on NK cells but not others.

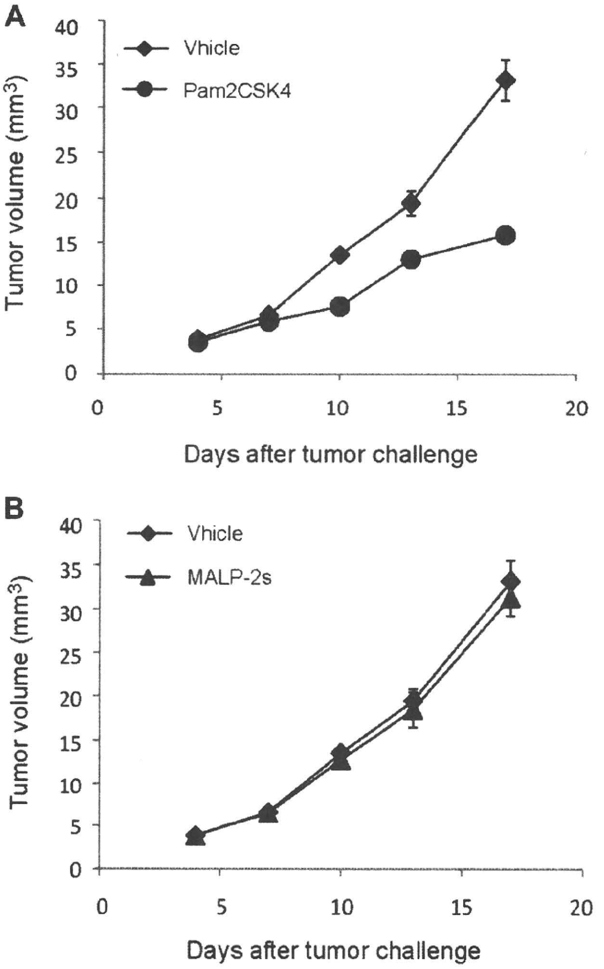

Fig. 5. MALP-2 fails to inhibit tumor growth *in vivo*. B16D8 cells were transplanted subcutaneously into mice at day 0. Mice (16 week-old, female) were treated with Pam2CSK4 (A) or MALP-2s (B) at day 0, 3, 7, 9, 13, 17 as described in Materials and methods. No tumor growth retardation was observed when MALP-2s was replaced with MALP-2f (not shown). Each group consists of $n = 4$. Surface diameters of the implanted tumors were measured. Mean ± SD are shown.

TLR2 in conjunction with TLR6 serves as an adjuvant receptor with potent cytokine-inducing ability, accompanied with up-regulation of IL-1β, IL-6, IL-12p40 and TNF-α. Apoptosis and NO production are also evoked through TLR2 stimulation [2]. The cytokine profile induced by MALP-2 indicates that macrophages and mDC differentiated from monocytes are targets for MALP-2 via their TLR2. TLR2 agonists facilitate induction of CTL and CD4 T cells against specific antigens [23,26]. In fact, M161Ag potently induces complement-associated inflammation [27] and maturation of immature mDC [16,28]. Although cytoplasmic sensors for bacterial lipoproteins may in part participate in the functional

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properties of MALP-2 [29], TLR2 agonistic activity in MALP-2 would involve cytokine-inducing properties but not anti-tumor function by intensifying *in vivo* NK activation.

Adjuvants are important for induction of vaccine immunity. Cancer immunotherapy has been developed using a variety of adjuvants. Intratumoral or intraperitoneal injection of MALP-2 has been attempted to induce suppression of pancreatic carcinoma in a mouse model [22]. In clinical trials, MALP-2 alone or in combination with gemcitabine was used for the treatment of unresectable pancreas carcinoma [6]. The rationale of this approach is based on the ability of MALP-2 to (1) act as a cytokine inducer [30], (2) activate murine as well as human DC to express co-stimulatory molecules [31], (3) induce a T-helper (Th) 1/2 response [32] and, most importantly, prolong survival in a mouse model of an orthotopic, syngeneic pancreas tumor [22]. Although Pam2 lipoproteins often induce an inhibitory cytokine IL-10 and regulatory T cells (Yamazaki S, unpublished data), no report mentioned the effect of these factors on MALP-2 adjuvant potential. We favored interpretation that the beneficial effects were due to immune activation, as we observed an increase in the expression of co-stimulatory molecules on lymphocytes, and cytotoxic T and NK cells infiltrating the tumor. However, our experiments with tumor-loaded mice showed that s.c. administration of MALP-2 confers no NK cell-mediated tumor regression on B16D8-implant mice. This unexpected result may be due to specific TLR2 agonistic properties of MALP-2 compared to peptidoglycan (that induces IL-12p70 in human mDC) [33] or instability of the lipid moiety of MALP-2. MALP-2 is degraded by two different mechanism in inflamed tissue: de-esterification and oxidation of the thioester bridge [6,34], thereby disappearing from the skin with a half time of ~20 h. Further modification will be required for *in vivo* use of this reagent.

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References

[1] T. Seya, M. Matsumoto, A lipoprotein family from *Mycoplasma fermentans* confers host immune activation through Toll-like receptor 2, Int. J. Biochem. Cell Biol. 34 (2002) 901–906.

[2] M. Matsumoto, M. Nishiguchi, S. Kikkawa, H. Nishimura, S. Nagasawa, T. Seya, Structural and functional properties of complement-activating protein M161Ag, a *Mycoplasma fermentans* gene product that induces cytokine production by human monocytes, J. Biol. Chem. 273 (1998) 12407–12414.

[3] M. Matsumoto, J. Takeda, N. Inoue, T. Hara, M. Hatanaka, K. Takahashi, S. Nagasawa, H. Akedo, T. Seya, A novel protein that participates in nonself discrimination of malignant cells by homologous complement, Nat. Med. 3 (1997) 1266–1270.

[4] K.L. Davis, K.S. Wise, Site-specific proteolysis of the MALP-404 lipoprotein determines the release of a soluble selective lipoprotein-associated motif-containing fragment and alteration of the surface phenotype of *Mycoplasma fermentans*, Infect. Immun. 70 (2002) 1129–1135.

[5] P.F. Mühlradt, M. Kiess, H. Meyer, R. Süssmuth, G. Jung, Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from *Mycoplasma fermentans* acting at picomolar concentration, J. Exp. Med. 185 (1997) 1951–1958.

[6] J. Schmidt, T. Welsch, D. Jäger, P.F. Mühlradt, M.W. Büchler, A. Märten, Intratumoral injection of the Toll-like receptor-2/6 agonist 'macrophage-activating lipopeptide-2' in patients with pancreatic carcinoma: a phase I/II trial, Br. J. Cancer 97 (2007) 598–604.

[7] D. Haller, P. Serrant, D. Granato, E.J. Schiffrin, S. Blum, Activation of human NK cells by staphylococci and lactobacilli requires cell contact-dependent costimulation by autologous monocytes, Clin. Diagn. Lab. Immunol. 9 (2002) 649–657.

[8] T. Miyake, Y. Kumagai, H. Kato, Z. Guo, K. Matsushita, T. Satoh, T. Kawagoe, H. Kumar, M.H. Jang, T. Kawai, T. Tani, O. Takeuchi, S. Akira, Poly I: C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways, J. Immunol. 183 (2009) 2522–2528.

[9] S. McCartney, W. Vermi, S. Gilfillan, M. Cella, T.L. Murphy, R.D. Schreiber, K.M. Murphy, M. Colonna, Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells, J. Exp. Med. 206 (2009) 2967–2976.

[10] T. Akazawa, M. Okuno, Y. Okuda, K. Tsujimura, T. Takahashi, M. Ikawa, M. Okabe, T. Ebihara, M. Shingai, N. Inoue, M. Tanaka-Okamoto, H. Ishizaki, J. Miyoshi, M. Matsumoto, T. Seya, Antitumor NK activation induced by the Toll-like receptor3-TICAM-1 (TRIF) pathway in myeloid dendritic cells, Proc. Natl. Acad. Sci. U S A 104 (2007) 252–257.

[11] Y. Akao, T. Ebihara, H. Masuda, Y. Saeki, K. Hazeki, O. Hazeki, M. Matsumoto, T. Seya, Enhancement of antitumor natural killer cell activation by orally administered Spirulina extract in mice, Cancer Sci. 100 (2009) 1494–1501.

[12] M. Azuma, R. Sawahata, Y. Akao, T. Ebihara, S. Yamazaki, M. Matsumoto, M. Hashimoto, K. Fukase, Y. Fujimoto, T. Seya, The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation, PLoS One 5 (2010) e12550.

[13] Y. Fujimoto, M. Hashimoto, M. Furuyashiki, M. Katsumoto, T. Seya, Y. Suda, K. Fukase, Innate immunostimulatory lipopeptides of *Staphylococcus aureus* as TLR2 ligands; Prediction with mRNA expression, chemical synthesis and immunostimulatory activities, Chembiochem 10 (2009) 2311–2315.

[14] O. Takeuchi, K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, S. Akira, Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components, Immunity 11 (1999) 443–451.

[15] Y. Saeki, K. Hazeki, M. Matsumoto, K. Toyoshima, H. Akedo, T. Seya, Correlation between metastatic potency and the down-regulation of E-cadherin in the mouse hepatoma cell lines G-1 and G-5, Oncol. Rep. 7 (2000) 731–735.

[16] M. Nishiguchi, M. Matsumoto, T. Takao, M. Hoshino, Y. Shimonishi, S. Tsuji, O. Takeuchi, S. Akira, K. Toyoshima, T. Seya, *Mycoplasma fermentans* lipoprotein M161Ag-induced cell activation is mediated by Toll-like receptor 2: role of N-terminal hydrophobic portion in its multiple functions, J. Immunol. 166 (2001) 2610–2616.

[17] D.M. Frucht, T. Fukao, C. Bogdan, H. Schindler, J.J. O'Shea, S. Koyasu, IFN-gamma production by antigen-presenting cells: mechanisms emerge, Trends Immunol. 22 (2001) 556–560.

[18] I. Fricke, D. Mitchell, J. Mittelstädt, N. Lehan, H. Heine, T. Goldmann, A. Böhle, S. Brandau, Mycobacteria induce IFN-gamma production in human dendritic cells via triggering of TLR2, J. Immunol. 176 (2006) 5173–5182.

[19] T. Akazawa, N. Inoue, H. Shime, K. Sugiura, K. Kodama, M. Matsumoto, T. Seya, Adjuvant engineering for cancer immunotherapy: development of

Please cite this article in press as: R. Sawahata et al., Failure of mycoplasma lipoprotein MALP-2 to induce NK cell activation through dendritic cell TLR2, Microbes and Infection (2011), doi:10.1016/j.micinf.2010.12.003

a synthetic TLR2 ligand with increased cell adhesion, Cancer Sci. 101 (2010) 1596–1603.

[20] O. Takeuchi, T. Kawai, P.F. Mühlradt, M. Morr, J.D. Radolf, A. Zychlinsky, K. Takeda, S. Akira, Discrimination of bacterial lipoproteins by Toll-like receptor 6, Int. Immunol. 13 (2001) 933–940.

[21] O. Takeuchi, S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R.L. Modlin, S. Akira, Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins, J. Immunol. 169 (2002) 10–14.

[22] C. Schneider, T. Schmidt, C. Ziske, K. Tiemann, K. Lee, V. Uhlinsky, P. Behrens, T. Sauerbruch, I. Schmidt-Wolf, P. Mühlradt, J. Schmidt, A. Märten, Tumoursuppression induced by macrophage activating lipopeptide in an ultrasound-guided syngeneic pancreatic carcinoma mouse model, Gut 53 (2003) 355–363.

[23] T. Akazawa, H. Masuda, Y. Saeki, M. Matsumoto, K. Takeda, S. Akira, K. Tsujimura, K. Kuzushima, T. Takahashi, I. Azuma, S. Akira, K. Toyoshima, T. Seya, Adjuvant-mediated tumor regression and tumor-specific cytotoxic response are impaired in MyD88-deficient mice, Cancer Res. 64 (2004) 757–764.

[24] M. Baratin, S. Roetynck, C. Lépolard, C. Falk, S. Sawadogo, S. Uematsu, S. Akira, B. Ryffel, J.G. Tiraby, L. Alexopoulou, C.J. Kirschning, J. Gysin, E. Vivier, S. Ugolini, Natural killer cell and macrophage cooperation in MyD88-dependent innate responses to *Plasmodium falciparum*, Proc. Natl. Acad. Sci. U S A 102 (2005) 14747–14752.

[25] J. Martinez, X. Huang, Y. Yang, Direct TLR2 signaling is critical for NK cell activation and function in response to vaccinia viral infection, PLoS Pathog. 6 (2010) e1000811.

[26] M.H. Wenink, K.C.M. Santegoets, J.C.A. Broen, L. van Bon, S. Abdollahi-Roodsaz, C. Popa, R. Huijbens, T. Remijn, E. Lubberts, P.L.C. M. van Riel, W.B. van den Berg, TR.D.J. Radstake, TLR2 promotes Th2/Th17 responses via TLR4 and TLR7/8 by abrogating the type I IFN amplification loop, J. Immunol. 183 (2009) 6960–6970.

[27] S. Kikkawa, M. Matsumoto, T. Sasaki, M. Nishiguchi, K. Tanaka, K. Toyoshima, T. Seya, Complement activation on *Mycoplasma fermentans* induced mycoplasma clearance from infected cells: Probing the organism with mAbs against M161Ag, Infect. Immun. 68 (2000) 1672–1680.

[28] Y. Nakao, K. Funami, S. Kikkawa, M. Taniguchi, M. Nishiguchi, Y. Fukumori, T. Seya, M. Matsumoto, Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells, J. Immunol. 174 (2005) 1566–1573.

[29] U. Buwitt-Beckmann, H. Heine, K.H. Wiesmüller, G. Jung, R. Brock, S. Akira, A.J. Ulmer, TLR1- and TLR6-independent recognition of bacterial lipopeptides, J. Biol. Chem. 281 (2006) 9049–9057.

[30] F. Rharbaoui, B. Drabner, S. Borsutzky, U. Winckler, M. Morr, B. Ensoli, P.F. Muhlradt, C.A. Guzman, The mycoplasma-derived lipopeptide MALP-2 is a potent mucosal adjuvant, Eur. J. Immunol. 32 (2002) 2857–2865.

[31] H. Weigt, P. Mühlradt, A. Emmendörfer, N. Krug, A. Braun, Synthetic derived mycoplasmal lipopeptide MALP-2 induces maturation and function of dendritic cells, Immunobiology 207 (2003) 1–11.

[32] F. Wittke, R. Hoffmann, J. Buer, I. Dallmann, K. Oevermann, S. Sel, T. Wandert, A. Ganser, J. Atzpodien, Interleukin 10 (IL-10): an immunosuppressive factor and independent predictor in patients with metastatic renal cell carcinoma, Br. J. Cancer 79 (1999) 1182–1184.

[33] S. Tsuji, M. Matsumoto, O. Takeuchi, S. Akira, I. Azuma, A. Hayashi, K. Toyoshima, T. Seya, Maturation of human dendritic cells by cell-wall skeleton of *Mycobacterium bovis* Bacillus Calmette-Guerin: involvement of Toll-like receptors, Infect. Immun. 68 (2000) 6883–6890.

[34] P.F. Mühlradt, M. Kiess, H. Meyer, R. Süssmuth, G. Jung, Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*, Infect. Immun. 66 (1998) 4804–4810.

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Reviews in Medical Virology

REVIEW

Antiviral responses induced by the TLR3 pathway

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Q1

SUMMARY

Antiviral responses are successively induced in virus-infected animals, and include primary innate immune responses such as type I interferon (IFN) and cytokine production, secondary natural killer (NK) cell responses, and final cytotoxic T lymphocyte (CTL) responses and antibody production. The endosomal Toll-like receptors (TLRs) and cytoplasmic RIG-I-like receptors (RLRs), which recognize viral nucleic acids, are responsible for virus-induced type I IFN production. RLRs are expressed in most tissues and cells and are primarily implicated in innate immune responses against various viruses through type I IFN production, whereas nucleic acid-sensing TLRs, TLRs 3, 7, 8 and 9, are expressed on the endosomal membrane of dendritic cells (DCs) and play distinct roles in antiviral immunity. TLR3 recognizes viral double-stranded RNA taken up into the endosome and serves to protect the host against viral infection by the induction of a range of responses including type I IFN production and DC-mediated activation of NK cells and CTLs, although the deteriorative role of TLR3 has also been reported in some virus infections. Here, we review the current knowledge on the role of TLR3 during viral infection, and the current understanding of the TLR3-signalling cascade that operates via the adaptor protein TICAM-1 (also called TRIF).

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INTRODUCTION

Mammalian cells possess several defense strategies against viral infection, of which, the type I interferon (IFN) system is most important for innate and adaptive antiviral responses [1,2]. Type I IFN induces an antiviral state in uninfected host cells by upregulating IFN-stimulated genes (ISGs) through IFN-α/β receptor signalling, and also activates innate and adaptive immune cells, such as dendritic cells (DCs), natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) [3]. Intrinsic double-stranded RNA (dsRNA) sensors, dsRNA-binding protein kinase R and 2′–5′ oligoadenylate synthetase, are both ISGs, which trigger the shutdown of protein translation and induce RNA degradation within virus-infected cells, respectively [4,5]. Recent progressive studies have demonstrated that the endosomal Toll-like receptors (TLRs) and cytoplasmic retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) are responsible for virus-induced type I IFN production [6–8]. These receptors recognize viral nucleic acids and induce type I IFN, inflammatory cytokine and chemokine production and DC maturation. TLR3 recognizes virus-derived dsRNA and its synthetic analogue, polyriboinosinic:polyribocytidylic acid (poly (I:C)) [9–11]. dsRNA is found in some virus particles as a viral genome and can be generated

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Abbreviations:
CT, C-terminal; CTL, cytotoxic T lymphocytes; CVB3, coxsackievirus group B serotype 3; dsRNA, double-stranded RNA; DC, dendritic cell; DUBA, deubiquitinating enzyme A; ECD, ectodomain; EMCV, encephalomyocarditis virus; HCV, hepatitis C virus; HSV-1, herpes simplex virus-1; IAV, influenza A virus; IFN, interferon; INAM, IRF-3-inducible NK activation molecule; ISG, IFN-stimulated gene; LRR, leucine-rich repeat; MCMV, murine cytomegarovirus; MDA5, melanoma differentiation associated gene 5; NAK, NF-κB activating kinase; NAP1, NAK-associated protein 1; NK, natural killer; NT, N-terminal; NTD, N-terminal domain of TICAM-1; pDC, plasmacytoid DC; poly(I:C), polyriboinosinic:polyribocytidylic acid; PVR, poliovirus receptor; RIG-I, retinoic acid inducible gene-I; RIP1, receptor-interacting protein 1; ss, single-stranded; TBK1, TANK-binding kinase 1; TICAM-1, TIR-containing adaptor molecule-1; TIR, Toll-IL-1 receptor; TLR, Toll-like receptor; RLR, RIG-I-like receptor; WNV, West Nile virus.

during the process of positive-stranded RNA virus and DNA virus replication [12]. TLR7 and TLR8 recognize virus-derived single-stranded (ss) RNA, while TLR9 recognizes non-methylated CpG-containing DNA that is found in some microbes [13–15]. Since these TLRs localize to the endosomal membranes of myeloid or plasmacytoid DCs (pDCs), they appear to detect extracellular viral nucleic acids released from infected cells or virus particles. However, the mechanism by which TLRs encounter virus-derived nucleic acids in endosomes remains to be determined. Interestingly, a recent report showed that TLR7-mediated IFN-α secretion by pDCs in response to ssRNA virus infection requires the transport of cytosolic viral RNA into the lysosome via the process of autophagy [16]. Whether this autophagy-dependent viral recognition is applicable to TLRs 3, 8 and 9 remains unclear.

By contrast, RLRs are expressed in most tissues and cells and detect viral nucleic acids in the cytoplasm. RIG-I recognizes viral RNA genomes bearing 5′-triphosphates and panhandle structures and also short-length dsRNAs [17–21], while melanoma differentiation-associated gene 5 (MDA5) detects long-length dsRNAs and poly(I:C) [22]. Studies using gene-disrupted mice and cells revealed that RIG-I is essential for the detection of various negative-stranded RNA viruses including influenza A virus (IAV), Sendai virus and vesicular stomatitis virus and a positive-stranded RNA virus, hepatitis C virus (HCV), whereas MDA5 plays a key role in sensing encephalomyocarditis virus, a member of *Picornavilridae* family [23–26]. Thus, multiple innate immune pathways are implicated in dsRNA responses and each pathway plays a distinct role in antiviral responses. In this review, we focus on TLR3, whose antiviral function has been controversial, but recent studies have demonstrated the critical role of the TLR3–TICAM-1 pathway in antiviral responses and the induction of adaptive immunity.

Expression and subcellular localization of TLR3

Human TLR3 mRNA has been detected in various tissues including the placenta, pancreas, lung, liver, heart and brain [27]. Interestingly, in the human central nervous system, TLR3 is expressed constitutively in neurons, astrocytes and microglia, suggesting a role in the response to viruses causing encephalopathy [28–30]. In immune cells, only myeloid DCs and macrophages express TLR3. The pDCs, which express TLR7 and TLR9 and secrete large amounts of IFN-α in response to viral infection, do not express TLR3 [31–35]. TLR3 is also expressed in fibroblasts and a variety of epithelial cells, including airway, corneal, cervical, biliary and intestinal cells [10,36–38], which are target sites of virus infection. TLR3 localizes both on the cell surface and endosomes in fibroblasts, macrophages and some of epithelial cell lines. Cell surface-expressed TLR3 participates in dsRNA recognition, as shown by the finding that an anti-human TLR3 monoclonal antibody (mAb) (TLR3.7) inhibits poly(I:C)-induced IFN-β production by fibroblasts [10]. By contrast, myeloid DCs only express TLR3 intracellularly [35]. Subcellular localization analysis showed that endogenous human TLR3 localizes to the early endosome but not to late endosomes/lysosomes in HeLa cells [39], while transfected human TLR3 predominantly localizes to multivesicular bodies in the mouse B-cell line Ba/F3, in which TLR3 was stably expressed at high levels. In any case, TLR3 signalling arises in the endosomal compartment, requiring endosomal maturation [35]. The 'linker' region consisting of 26 a.a. between the transmembrane domain and the Toll-IL-1 receptor (TIR) domain of TLR3, determines intracellular localization of TLR3 [40,41]. An unidentified molecule associating with the linker region may regulate the endosomal retention of TLR3 in myeloid DCs.

Notably, TLR3 expression is upregulated by viral infection and the exogenous addition of poly(I:C) or type I IFN [42]. The IFN-responsive element is located at approximately −30 bp in the human *TLR3* promoter region [43,44].

Recognition of dsRNA by TLR3

TLR3 recognizes dsRNA through its ectodomain (ECD), which induces receptor dimerization required for adaptor-mediated signal transduction [45]. TLR3 consists of an ECD formed by 23 leucine-rich repeats (LRRs) and N- and C-terminal flanking regions, known as the LRR N-terminal (LRR-NT) and C-terminal (LRR-CT) regions, the transmembrane domain and the cytoplasmic TIR domain [46] (Figure 1A). TLR3–ECD possesses 15 putative carbohydrate-binding motifs. Structural analyses

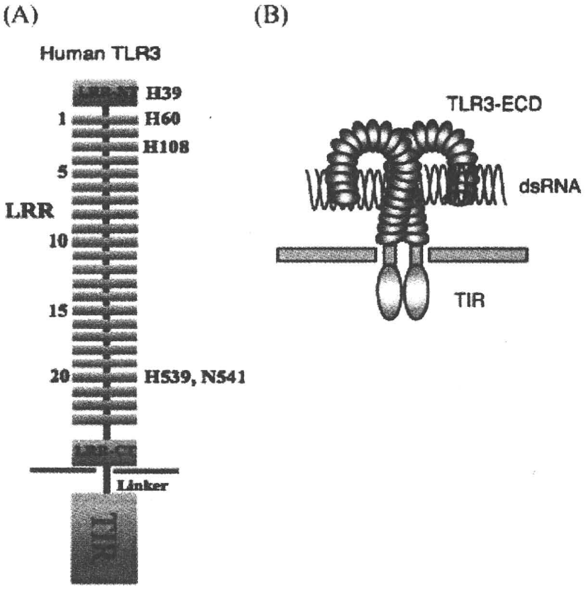

Figure 1. (A) Schematic structure of human TLR3. TLR3 is a type I transmembrane protein of 904 a.a. TLR3 consists of an ECD formed by 23 LRRs and N- and C-terminal flanking regions (LRR-NT and LRR-CT), the transmembrane domain, cytoplasmic linker region and the TIR domain. H539 and N541 in TLR3-LRR20, H39 in the LRR-NT, H60 in LRR1 and H108 in LRR3 are essential for dsRNA-binding. (B) Model of the dsRNA–TLR3-signalling complex. dsRNA interacts with both an N- and a C-terminal binding site on the glycan-free surface of each TLR3–ECD, which are located on opposite sides of the dsRNA. The proximity of the two C-termini facilitates the dimerization of the cytoplasmic TIR domains

of human TLR3–ECD revealed that the LRRs form a large horseshoe-shaped solenoid of which one face is largely masked by carbohydrate, while the other face is unglycosylated [47,48]. By point mutation analysis, Bell *et al.* [49] demonstrated that the His539 and Asn541 residues in TLR3–LRR20, located on the glycan-free lateral face, are critical amino acids for dsRNA binding and signalling. Wild-type TLR3–ECD protein directly binds poly(I:C) at pH7.6, while mutant proteins H539E and N541A fail to bind poly(I:C). Based on the observation that an acidic pH (pH 6.0 and below) is required for TLR3 recognition of dsRNA, the N-terminal conserved histidine residues, His39 in the LRR-NT, His60 in LRR1 and His108 in LRR3, were identified as a second binding site for dsRNA [50,51]. Protonation of these imidazole groups under acidic conditions, such as those found in endosomes, appears to generate an ionic interaction between the histidine residues and the negatively charged phosphate backbone of dsRNA.

In addition, Leonard *et al.* [52], showed that TLR3–ECD binds as a dimer to 40–50 bp length of dsRNA, and multiple TLR3–ECD dimers bind to long dsRNA strands. Binding affinities increase with both buffer acidity and dsRNA length. At the pH within early endosomes (~6.0–6.5), >90-bp length of dsRNA is required to form a stable complex with TLR3. However, at the pH within late endosomes (~5.5 and below), 40–50-bp length of dsRNA forms stable complex with dimeric TLR3, suggesting that dsRNA-induced TLR3-mediated signalling depends on the length of the dsRNA and the TLR3 localization site [52]. Finally, structural analysis of the complex of two mouse TLR3–ECDs and one 46-bp dsRNA oligonucleotide revealed that dsRNA interacts with both an N- and a C-terminal binding site on the glycan-free surface of each mTLR3–ECD, which are located on opposite sides of the dsRNA [53] (Figure 1B). The dsRNA in the complex retains a typical A-form DNA-like structure. dsRNA has been predicted to adopt a right-handed A-form helix with 11 bp per helical turn and a 28 Å helical pitch [54]. Therefore, two helical turns would fit between the N- and C-terminal binding sites of TLR3 [53]. In addition, the two LRR-CT domains are brought into proximity and form a series of protein–protein interactions, which facilitate the dimerization of the cytoplasmic TIR domain. Funami *et al.* [40], reported that the Phe732, Leu742 and Gly743 residues in the TLR3 cytoplasmic linker region are essential for TLR3 signalling, suggesting that the linker region controls the dimerization of the TLR3–TIR domain.

TLR3–TICAM-1-signalling pathway

TLR3 mediates signalling via an adaptor protein, TIR-containing adaptor molecule-1 (TICAM-1; also called TRIF) [55,56] (Figure 2A). TICAM-1 activates the transcription factors IRF-3, NF-κB and AP-1, leading to the induction of type I IFN, cytokine/chemokine production and DC maturation, which then enables the activation of NK cells and CTLs. TLR3 also associates with c-Src tyrosine kinase on endosomes in response to dsRNA [57]. The Src kinase inhibitor markedly inhibits dsRNA-elicited phosphorylation of Akt, a downstream target of phosphatidylinositol 3-kinase (PI3-K). In addition, PI3-K is required for full phosphorylation and activation of IRF-3 by dsRNA [58]. The precise role of c-Src in IRF-3 activation via the PI3-K-Akt pathway requires further elucidation.

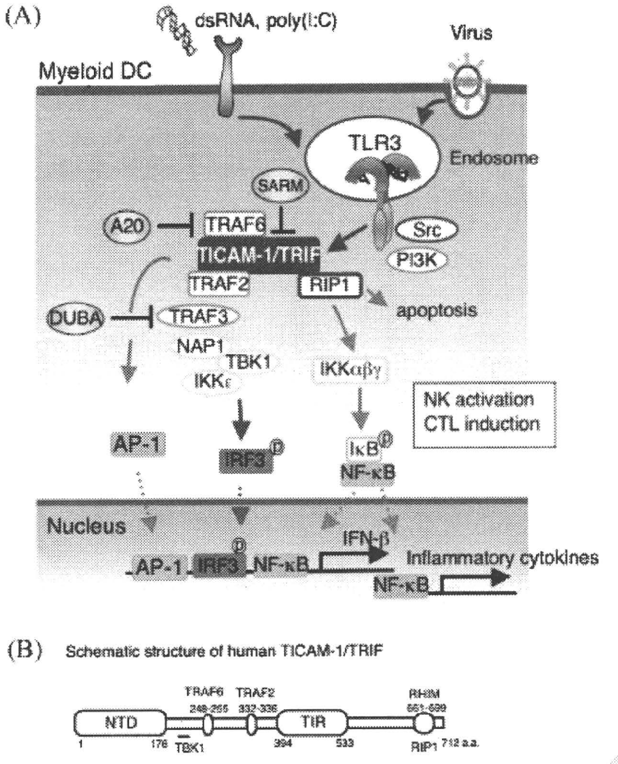

Figure 2. (A) TLR3–TICAM-1-signalling pathway. In myeloid DCs, TLR3 is expressed in the endosomal compartments and recognizes extracellular viral dsRNA and its synthetic analogue poly(I:C). Once TLR3 is dimerized by dsRNA, it recruits the adaptor protein TICAM-1/TRIF that activates the transcription factors, IRF3, NF-κB and AP-1. RIP1 associates with TICAM-1 via the PHIM domain in the C-terminal region and acts as an NF-κB activator and apoptosis mediator in TICAM-1-mediated signalling. TRAF3 and NAP1 participate in the recruitment and activation of the IRF-3 kinases TBK1 and IKKε. Phosphorylated IRF-3 translocates into the nucleus and together with NF-κB and AP-1 induces IFN-β gene transcription. The TICAM-1-mediated AP-1 activation pathway is unclear. (B) Schematic structure of human TICAM-1/TRIF. N-terminal domain (NTD) (1–176 a.a.), TIR domain (394–533 a.a.), RHIM domain (661–699 a.a.), TRAF6-binding site (248–256 a.a.), TRAF2-binding site (332–336 a.a.) and TBK1-binding site (under line) are shown.

TICAM-1 consists of an N-terminal region, a TIR domain and a C-terminal region (Figure 2B). The TIR domain of TICAM-1 is essential for binding to the TIR domain of TLR3 and also to the TLR4 adaptor TICAM-2 (also called TRIF-related adaptor molecule) [59,60]. TICAM-1 is expressed at a low level in most tissues and cells and is diffusely localized in the cytoplasm of resting cells [39]. When endosomal TLR3 is activated by dsRNA, TICAM-1 transiently co-localizes with TLR3, then dissociates from the receptor and forms speckled structures that co-localize with downstream-signalling molecules [39]. Homo-oligomerization through the Pro434 residue in the TIR domain and the C-terminal region is essential for TICAM-1-mediated activation of NF-κB and IRF-3 [61]. Once TICAM-1 is oligomerized, the serine-threonine kinases, TANK-binding kinase 1 (TBK1; also called NAK or T2K) and IkB kinase-related kinase-ε (IKK-ε; also called IKK-i), are activated and phosphorylate IRF-3 [62,63]. The ubiquitin ligase of the TRAF family members, TRAF2, TRAF3 and TRAF6, are downstream-signalling molecules of TICAM-1. TRAF2 and TRAF6 directly bind to the N-terminal region of TICAM-1 [64,65] (Figure 2B). The Lys63-linked autoubiquitination of TRAF3 is required for IRF3 activation [66,67]. Furthermore, NF-κB-activating kinase (NAK)-associated protein 1 (NAP1) participates in the recruitment of IRF-3 kinases to the N-terminal region of TICAM-1 [68]. Although both TRAF3 and NAP1 associate with oligomerized TICAM-1 and serve as a critical link between TICAM-1 and downstream IRF-3 kinases, there is no evidence that they bind directly to TICAM-1. Interestingly, recent reports showed that direct binding of TBK1 to TICAM-1 is necessary for IRF-3 activation [69]. The Leu194 residue in the N-terminal region is critical for TBK1 binding to TICAM-1. In addition, the Ser189, Arg195 and Ser196 residues are involved in TBK1–TICAM-1 binding.

The N-terminal 176 a.a. of TICAM-1 form a protease-resistant structural domain, designated NTD (Figure 2B). Because the crucial amino acids for TRAF2-, TRAF6- and TBK1-binding reside between the NTD and the TIR domain, naive TICAM-1 may have a closed conformation that covers these binding sites. Indeed, protein–protein interaction analysis revealed that the NTD interacts with the N-terminus of TICAM-1–TIR [69]. Thus, the NTD folds into the TIR domain structure to maintain the naive conformation of TICAM-1. Upon stimulation of TLR3 or TLR4, TICAM-1 oligomerizes through the TIR domain and the C-terminal region, possibly breaking the intramolecular association and inducing a conformational change that allows TBK1 access to TICAM-1.

Whereas the N-terminal region is crucial for TICAM-1-mediated IRF-3 activation, the C-terminal region of TICAM-1 is involved in NF-κB activation and apoptosis. Receptor-interacting protein 1 (RIP1), a kinase containing a death domain, associates with

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TICAM-1 via the RIP homotypic interaction motif domain in the C-terminal region and acts as an NF-κB inducer and apoptosis mediator in TICAM-1-mediated signalling [70–72]. TRAF6 has also been implicated in NF-κB activation by TICAM-1 in a cell-type-dependent manner [64,73].

TLR3–TICAM-1-mediated signalling is negatively regulated by a fifth TIR adaptor protein SARM [74]. SARM and TICAM-1 have been shown to interact and SARM strongly suppresses NF-κB activation, as well as IRF-3 activation by TICAM-1. Moreover, deubiquitinating enzyme A (DUBA) negatively regulates TLR3-mediated type I IFN production. DUBA selectively cleaves the Lys63-linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream-signalling molecules [75]. In addition, the ubiquitin-modifying enzyme A20 inhibits TICAM-1-mediated NF-κB activation by deubiquitinating TRAF6 [76]. However, the precise mechanisms by which TRAF3 and TRAF6 are ubiquitinated and their interaction with downstream-signalling molecules are unknown.

Antiviral function of TLR3

The role of TLR3 in viral infection is complex (Table 1). Studies in TLR3-deficient (TLR3$^{-/-}$) mice showed that the immune response to different viruses, including lymphocytic choriomeningitis virus (an ambisense RNA virus), vesicular stomatitis virus (a negative-stranded RNA virus), murine cytomegarovirus (MCMV, a dsDNA virus) and reovirus (a dsRNA virus), was unaffected in these mutant mice compared with wild-type mice [77].

By contrast, Hardarson *et al.* [78] reported that TLR3 is important in host defense against encephalomyocarditis virus (EMCV, a positive sense ssRNA virus belonging to the *Picornaviridae* family). When mice were inoculated intraperitoneally with 50 plaque-forming units EMCV, TLR3$^{-/-}$ mice were more susceptible to EMCV infection and had a significantly high viral load in the heart compared with wild-type mice. Opposing to these data, Kato *et al.* [24] showed that MDA5 but not TLR3 plays an important role in host defense against EMCV infection, when mice were infected with 100 plaque-forming units EMCV intraperitoneally. It is unclear why these different results were obtained from similar EMCV infection studies.

Table 1. The role of[Q2] TLR3 in viral infection

	References
Protection	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[84]
Picornaviridae [+, ss]	
Encephalomyocarditis virus (EMCV)	[78]
Poliovirus	[79,80]
Coxsackievirus group B serotype 3 (CVB3)	[82]
Herpesviridae [dsDNA]	
Murine cytomegarovirus (MCMV)	[90]
Herpes simplex virus 1 (HSV-1)	[101]
Deterioration	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[83]
Orthomyxoviridae [−, ss]	
Influenza A virus (IAV)	[88]
Bunyaviridae [−, ss]	
Phlebovirus	[89]

More recently, the essential role of the TLR3–TICAM-1 pathway in protection from poliovirus infection, a virus belonging to the *Picornaviridae* family, has been demonstrated [79,80]. Poliovirus receptor (PVR)-transgenic/TICAM-1-deficient mice are more susceptible than PVR-transgenic mice to intraperitoneal or intravenous inoculation with a low titre of poliovirus [79,80]. Forty-eight hours after infection, virus titres in serum dramatically increased and mortality greatly decreased compared with PVR-transgenic or PVR-transgenic/IPS-1 (RLR adaptor)-deficient mice. It is well known that in cultured mammalian cells, poliovirus infection results in inhibition of cellular protein synthesis so-called 'shut-off' event [81]. Therefore, mRNA upregulation of RIG-I and MDA5 by type I IFN does not link to protein synthesis at an early stage of virus infection. Thus, it appears that the inhibitory effects of viral multiplication on host cells depend on the TLR3–TICAM-1 pathway, but not the RLR–IPS-1 pathway.

Negishi *et al.* [82] showed that TLR3$^{-/-}$ mice are more vulnerable to coxsackievirus group B serotype 3 (CVB3, a virus belonging to the *Picornaviridae* family) than wild-type mice, in terms of higher mortality and acute myocarditis. The expression of IL-12p40, IL-1β and IFN-γ mRNAs, but not IFN-β mRNA, was impaired in the hearts of CVB3-infected TLR3-deficient mice compared with those of wild-type mice infected with CVB3. By contrast, expression of TLR3 by transgene protects mice from lethal CVB3 infection and hepatitis even in the absence of type I IFN signalling. Antibody blocking studies revealed that TLR3–TICAM-1-dependent type II IFN (IFN-γ) production is critical for host defense against CVB3 infection [82].

Wang *et al.* [83] demonstrated that TLR3 is involved in the viral pathogenesis of West Nile virus (WNV, a positive-stranded RNA virus). TLR3$^{-/-}$ mice showed impaired cytokine production and enhanced viral loads in the periphery, whereas in the brain, the viral load, inflammatory responses and neuropathology were reduced compared with wild-type mice [83]. TLR3-mediated peripheral inflammatory cytokine production is critical for disruption of the blood–brain barrier, which facilitates viral entry into the brain causing lethal encephalitis. Therefore, TLR3$^{-/-}$ mice are more resistant to lethal WNV infection. In contrast, Daffis *et al.* [84] reported the protective role of TLR3 in sublethal WNV infection. The absence of TLR3 enhances WNV mortality in mice and increases viral burden in the brain after inoculation with the pathogenic New York strain of WNV, although there are little differences in WNV-specific antibody responses, CD8$^+$ T-cell activation, blood–brain barrier permeability and IFN-α/β induction in draining lymph nodes and serum, between wild-type and TLR3$^{-/-}$ mice [84]. The reason why TLR3 shows the opposite function against WNV infection remains to be determined.

In other RNA viral infections such as respiratory syncytial virus, IAV and phlebovirus (all negative-stranded RNA viruses), TLR3-dependent inflammatory cytokine and chemokine production also appears to affect virus-induced pathology and host survival [85–89]. TLR3$^{-/-}$ mice infected with IAV exhibited reduced inflammatory mediators, leading to increased survival [88]. It is notable that experimental conditions using high viral doses may lead to the over-production of inflammatory cytokines and chemokines. However, what type of TLR3-expressing cells that respond to virus-derived dsRNA *in vivo* has not been shown in these studies.

Cellular immunity induced by the TLR3–TICAM-1 pathway

In addition to type I IFNs, CTLs and NK cells are also principal effector cells in antiviral immunity. The contribution of TLR3 to antiviral responses has been shown in MCMV infection [90], during which virus clearance is partly dependent on NK cell activation. TLR3$^{-/-}$ mice are hypersusceptible to MCMV infection. Cytokine (type I IFN, IL-12p40 and IFN-γ) production, and NK cell and NKT cell activation are impaired in TLR3$^{-/-}$ mice compared with wild-type mice.

Selective TLR3 expression in myeloid DCs but not in pDCs raises the possibility that TLR3 also plays a key role in the antiviral response by induction of adaptive immune responses rather than primary IFN-α/β production (Table 2). Myeloid DCs are the most effective professional antigen-presenting cells, possessing several antigen processing and transporting pathways [91,92]. One of the most notable features of myeloid DCs is the cross-presentation of exogenous antigens to CD8$^+$ T cells. This pathway is important for effective host CTL induction against viruses that do not directly infect DCs. Among the myeloid DC subsets, the splenic CD8α$^+$ DC subset in mice and the CD141(BDCA3)$^+$DNGR-1(CLEC9A)$^+$ DC subset in humans highly express TLR3 and display a superior capacity for cross-presenting apoptotic and necrotic cell antigens after TLR3 stimulation [93–97]. Using TLR3-deficient mice, Schultz *et al.* [98] clearly showed that TLR3 plays an important role in cross-priming. Mouse CD8α$^+$ DCs are activated by phagocytosis of apoptotic bodies from virally infected cells or cells containing poly(I:C) in a TLR3-dependent manner. Furthermore, immunization with virally infected cells or cells containing poly(I:C), both carrying ovalbumin antigen, induces ovalbumin-specific CD8$^+$ T-cell responses, which are largely dependent on TLR3-expressing DCs [98]. In many cases, virally infected cells produce IFN-α/β which activates DCs to

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

	DC subset	TLR3	TLR7	TLR8	TLR9	References
Human	Myeloid DC					
	MoDC	+	−	+	−	[31–35]
	$CD11c^+CD1c^+DC$	+	−	+	−	[34,35,94,95]
	$CD141^+CLEC9A^+DC$	++	−	+	−	[94,95]
	Plasmacytoid DC	−	+	−	+	[34,35]
Mouse	Myeloid DC					
	BMDC	+	−	−	+	[95]
	$CD8\alpha^+$ DC	++	−	−	+	[93,95]
	Plasmacytoid DC	−	+	−	+	[93,95]

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

promote $CD8^+$ T-cell cross-priming [99]. Thus, both TLR3- and IFN-α/β-mediated signalling are likely implicated in licensing DCs for the cross-priming of $CD8^+$ 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 $CD141^+$ 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 $CD8\alpha^+$ DCs and human $CD141^+$ 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 $CD141^+$ DCs and mouse $CD8\alpha^+$ 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-α 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-α 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 activation 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-β 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 anti-viral 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-κB 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). *ADDR* 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 biliary 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.

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-β 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-β. *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-κB 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-κB 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-β (TRIF) associates with TNFR-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-κB 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-β (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) (abstract^{Q3}).

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 α+ DC correlates with unresponsiveness to imidazoquinolines. *European Journal of Immunology* 2003; **33**: 827–833.

94. Jongbloed SL, Kassianos AJ, McDonald KJ, *et al.* Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *Journal of Experimental Medicine* 2010; **207**: 1247–1260.

95. Poulin LF, Salio M, Griessinger E, *et al.* Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8α+ 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 CD11c+ CD141+ cells as homologues of mouse CD8+ 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 CD8α+ 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 CD8+ 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 CD8α+ DCs and human BDCA3+ 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 alpha+ 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-β production. *Journal of Immunology* 2008; **181**: 5522–5529.

110. Okahira S, Nishikawa F, Nishikawa S, Akazawa T, Seya T, Matsumoto M. Interferon-β 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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Strain-to-strain difference of V protein of measles virus affects MDA5-mediated IFN-β-inducing potential

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ABSTRACT

Laboratory-adapted and vaccine strains of measles virus (MV) induce type I interferon (IFN) in infected cells to a far greater extent than wild-type strains. We investigated the mechanisms for this differential type I IFN production in cells infected with representative MV strains. The overexpression of the wild-type V protein suppressed melanoma differentiation-associated gene 5 (MDA5)-induced IFN-β promoter activity, while this was not seen in A549 cells expressing CD150 transfected with the V protein of the vaccine strain. The V proteins of the wild-type also suppressed poly I:C-induced IFN regulatory factor 3 (IRF-3) dimerization. The V proteins of the wild-type and vaccine strain did not affect retinoic acid-inducible gene 1 (RIG-I)- or toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1)-induced IFN-β promoter activation. We identified an amino acid substitution of the cysteine residue at position 272 (which is conserved among paramyxoviruses) to an arginine residue in the V protein of the vaccine strain. Only the V protein possessing the 272C residue binds to MDA5. The mutation introduced into the wild-type V protein (C272R) was unable to suppress MDA5-induced IRF-3 nuclear translocation and IFN-β promoter activation as seen in the V proteins of the vaccine strain, whereas the mutation introduced in the vaccine strain V protein (R272C) was able to inhibit MDA5-induced IRF-3 and IFN-β promoter activation. The other 6 residues of the vaccine strain V sequence inconsistent with the authentic sequence of the wild-type V protein barely affected the IRF-3 nuclear translocation. These data suggested that the structural difference of vaccine MV V protein hampers MDA5 blockade and acts as a nidus for the spread/amplification of type I IFN induction. Ultimately, measles vaccine strains have two modes of IFN-β-induction for their attenuation: V protein mutation and production of defective interference (DI) RNA.

1. Introduction

Innate immunity is the first line of defense against virus infection, and the most powerful antiviral agent possessed by the host immune system is interferon (IFN). Expression of type I IFN in host cells induces a set of IFN-inducible genes which efficiently suppress viral replication and spread (Pichlmair and Reis, 2007). Host cells usually terminate virus replication in response to IFN induction. Recent studies elucidated the mechanism by which type I IFN is induced and found that it senses virus patterns such as 5′-triphosphate (5′-3P) and stem-loops or double-stranded RNA (dsRNA) (Takeuchi and Akira, 2008). dsRNA specifically is present in several forms: viral genomes, single-stranded RNA virus replication intermediates, DNA virus symmetrical transcription products, defective viral particles and debris from lysed cells (Bowie and Fitzgerald, 2007). These viral products all present patterns that activate the IFN system. In fact, extracellular dsRNA is sensed by endosomal Toll-like receptor 3 (TLR3), and intracellular dsRNA is detected by cytoplasmic RNA helicase retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Takeuchi and Akira, 2008). TLR3 recruits the adaptor, toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1, also named TRIF) (Oshiumi et al., 2003). RIG-I and MDA5 signal through IFN-β promoter stimulator 1 (IPS-1). These adaptor molecules activate kinase TANK-binding kinase I (TBK1), inhibitor of κB kinase ε (IKKε) and NAK-associated protein 1 (NAP-1) (Sasai et al., 2006a). These complexes then phosphorylate IFN regulatory factor 3 (IRF-

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3) and IRF-7, promoting their dimerization, nuclear translocation and transcription of IFN-stimulated genes (ISGs), such as ISG56, as well as IFN and other cytokines (Medzhitov, 2007; Platanias, 2005). On the other hand, secreted IFNs bind to the IFN-α/β receptor on the surface of adjacent cells and activate the Janus kinase–signal transducer and activator of transcription (JAK/STAT) signaling pathway, which amplifies IFN induction and stimulates transcription of a variety of antiviral genes (Samuel, 2001). Many viruses encode specific proteins to inhibit IFN induction or the JAK/STAT pathway (Katze et al., 2002; Sen, 2001). The V protein of measles virus (MV) blocks the IFN-inducing pathway mediated by MDA5 and the JAK/STAT pathway (Ohno et al., 2004; Nakatsu et al., 2008). The C protein of MV acts as a regulator of viral RNA synthesis, thereby acting indirectly to suppress IFN induction (Nakatsu et al., 2008).

It has been reported that wild-type measles strains barely induce type I IFN (Naniche et al., 2000; Shingai et al., 2007). The levels of IFN protein or mRNA are lower than the detection limit in cells infected with wild-type MV, while higher levels of IFN are detectable in cells infected with vaccine strains. Although the mechanism behind the strain-to-strain differences in IFN-inducing potential remain unclear, an early report suggested that a laboratory strain, strain Edmonston (ED), possesses a unique V protein with low suppression of IFN-α/β receptor (IFNAR)-amplifiable IFN induction (Ohno et al., 2004). We previously reported that vaccine/laboratory strains harbor defective interference (DI) RNA which activates RIG-I and/or MDA5. Type I IFN is efficiently yielded by DI RNA during viral RNA replication (Shingai et al., 2007). We found that the majority of measles vaccine and laboratory-adapted strains possess DI RNA. However, the IFN-inducibility of attenuated MV strains does not always correlate with the presence of DI RNA. Therefore, the mechanisms by which the primary IFN-inducing activity by RIG-I/MDA5 is impaired during wild-type measles infection still remain unexplained.

In this study, using wild-type and DI-negative attenuated measles strains, we investigated the predominate mechanisms that act on the host IFN system to modulate IFN production. We identified amino acid differences between the V proteins of the attenuated ED strain and wild-type MV, and found that the cysteine residue at position 272 (272C) was required for suppression of MDA5-induced type I IFN production.

2. Materials and methods

2.1. Cell culture and reagents

The human lung epithelial cell line (A549), A549/CD150, African green monkey kidney cell line (Vero), Vero/CD150 and HEK293FT cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (Tanabe et al., 2003). HeLa cells were cultured in Eagle's MEM with 10% heat-inactivated FCS and L-glutamine. For establishing CD150-expressing A549 and Vero cell lines, pCNX2-huCD150 was introduced into cell lines using FugeneHD (Roche) according to the manufacturer's protocol. Twenty-four hours after transfection, the neomycin analog G418 (Sigma–Aldrich) was added to the medium at the final concentration of 1.4 mg/ml or 0.6 mg/ml for Vero or A549 cells. During selection, G418-containing medium was changed once every 4 days. G418-resistant, stably transfected clones were propagated for the study of surface expression of CD150 by flow cytometer. The following antibodies were obtained commercially: anti-FLAG (Sigma–Aldrich); anti-Myc (Santa Cruz); anti-IRF-3 (IBL). Alexa Fluor 488- and Alexa Fluor 568-conjugtaed secondary antibodies were from Invitrogen Life Technologies. Polyriboinosinic/polyribocytidylic acid (polyI:C) was from Amersham Biosciences.

2.2. Plasmids

Complementary DNAs of human TICAM-1, MDA5, RIG-I, V and C were cloned in our laboratory by RT-PCR and ligated into the cloning site of the expression vector, pEF-BOS, pcDNA4 Myc-HisA and pCMV10-FLAG (Funami et al., 2008). Mutations were introduced by site-directed mutagenesis using PCR. All constructs were confirmed by sequencing.

2.3. Virus preparation and titration

Nagataha (NV) and Edmonston (ED) strains were obtained from Dr. S. Ueda (the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and University of Washington (Seattle, WA), respectively. Ichinose (IC)-B was provided from Dr. F. Kobune (National Institutes of Health, Tokyo, Japan) (Kubune et al., 1990). Masusako (MS) was propagated in our laboratory (Kurita-Taniguchi et al., 2000; Murabayashi et al., 2002). NV, ED and MS strains were maintained in Vero/CD150 cells in our laboratory (Shingai et al., 2007). IC-B strain was maintained in B95a cells. Virus titer was determined as PFUs on Vero/CD150 and the multiplicity of the infection (MOI) of each experiment was calculated based on this titer (Kubune et al., 1990).

2.4. RT-PCR and real-time PCR

Total RNA was prepared using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. RT-PCR was carried out using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. The following oligonucleotides were used for human GAPDH: 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′; and for MV-H: 5′-CCCTTATCAACGGATGATCC-3′ and 5′-GTGATCAATGGCCCCAATCC-3′; and for q-PCR human β-actin: 5′-CCTGGCACCCAGCACAAT-3′ and 5′-GCCGATCCACACGGAGTACT-3′; and for *q*-PCR human IFN-β: 5′-CAATTGCTTGGATTCCTACAAAG-3′ and 5′-TATTCAAGCCTCCCATTCAATTG-3′. IFN-β mRNA were normalized to β-actin and fold inductions of transcripts were calculated using the ddCT method relative to unstimulated HeLa cells.

2.5. RT-PCR amplification of cDNA from 5′ copy-back DI RNAs

We modified the RT-PCR amplification protocol of Calain et al. (1992), where the copy-back DI RNAs were amplified using two set of MV primers (for 5′ copy-back DIs, JM396; 5′-TATAAGCTTACCAGACAAAGCTGGGAATAGAAACTTCG-3′/JM403; 5′-CGAAGATATTCTGGTGTAAGTCTAGTA-3′, and for standard genome, JM396/JM402; 5′-TTTATCCAGAATCTCAARTCCGG-3′) (Sidhu et al., 1994; Whistler et al., 1996). Viral RNA from the culture supernatant was extracted with QIAamp Viral RNA Mini kit (Qiagen). Total RNA from viral-infected cells was extracted with TRIzol Reagent following the manufacturer's instructions. RT-PCR was carried out using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. The PCR-amplified products were confirmed by sequencing.

2.6. Reporter gene assay

Cells were seeded onto 24-well plates and transfected with various amounts of expression vectors, the reporter gene, and the phRL-TK control plasmid using FuGene HD (Roche) according to the manufacturer's instructions. After 24 h, the cells were harvested in 100 μl lysis buffer. The luciferase activity was measured using Dual-

Luciferase Reporter assay systems (Promega) and was shown as the means ± S.D. of three experiments.

2.7. *Native PAGE, SDS-PAGE, Western blotting, and immunoprecipitation assay*

Cells were solubilized in the lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% NP-40, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM Na_3VO_4) on ice for 30 min and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants were separated by SDS-PAGE, and the gel was transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with Tris-buffered saline (TBS) pH 8.0 containing 5% skim milk, immunoblotted with specific antibodies, and visualized with the appropriate horseradish peroxidase-conjugated secondary antibodies using the ELC plus Western Blotting Detection System (Amersham Pharmacia). For detection of IRF3-dimerization, whole cell extracts were subjected to 7.5% polyacrylamide gel Native (Daiichi Pure Chemicals). For immunoprecipitation, cells were lysed in the Triton X-100 lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM Na_3VO_4) and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants were incubated with anti-Myc antibody and protein G-Sepharose (Amersham Pharmacia) for overnight at 4 °C. The immunoprecipitates were collected by centrifugation, washed 4 times in the lysis buffer, and then analyzed by SDS-PAGE.

2.8. *Confocal microscopy*

HeLa cells (2.5×10^4 cells/well) were plated on a micro cover glass (Matsunami Glass) in 12-well plate. The following day, cells were transfected with the indicated plasmids using FuGENE HD (Roche). The total amounts of DNA were kept constant by adding empty vector. After 24 h, cells were fixed in acetone and blocked in PBS containing 1% BSA and then labeled with the indicated primary antibodies for 1 h at room temperature. Alexa Fluor 488- or Alexa Fluor 594-conjugtaed secondary antibodies were used for the visualizing proteins detected by the primary antibodies. For nucleus staining, cells were treated with DAPI in PBS. After all staining procedures were finished, micro cover glasses were mounted onto a slide glass using PBS containing 2.3% DABCO and 50% glycerol. Cells were visualized at 63× magnification under an LSM510 META microscope (Zeiss).

2.9. *Statistical analysis*

The statistical significance was analyzed using Student's *t*-test. *p* values < 0.05 were considered significant.

3. Results

3.1. *Laboratory adapted strain ED induces IFN-β mRNA in A549/CD150 cells*

We tested whether MV induced the expression of IFN-β mRNA in infected A549/CD150 cells and found that laboratory-adapted strain ED induced IFN-β mRNA expression, whereas IFN-β mRNA was virtually undetectable in wild-type strain MS-infected cells (Fig. 1A). To confirm the efficiency of virus infection, we measured MV-H mRNA levels by RT-PCR (Fig. 1B). The MV-H mRNA level in MS-infected cells was comparable to that found in ED-infected cells. Our previous report showed that DI RNA in MV isolates is a crucial determinant for high IFN induction (Shingai et al., 2007). However, no amplifiable 5′ copy-back DI RNA was detected in the MV culture supernatants (Fig. 1C), suggesting that the ED and MS strains used in this study, do not contain 5′ copy-back DI RNA. Thus, in this DI RNA-negative ED strain, a factor other than DI RNA is implicated in the induction of IFN-β mRNA.

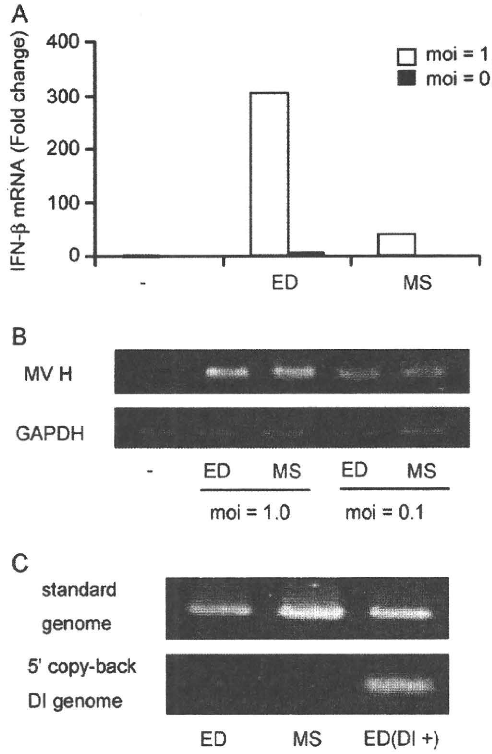

Fig. 1. ED strain induces IFN-β mRNA expression in the infected cells. (A) A549/CD150 cells were infected with mock, ED (vaccine strain) or MS (wild-type strain) at MOI = 0.1 or 1. After 12 h, RNA samples were collected and mRNAs of IFN-β and β-actin were measured by real-time PCR. The value for IFN-β mRNA expression was normalized to that of β-actin mRNA. Fold induction against control medium is shown. (B) MV-H mRNA level was determined by RT-PCR. (C) RT-PCR amplification of 5′ copy-back DI RNA from MV culture supernatants. RT-PCR was performed using standard genome-specific primers or DI-specific primers.

3.2. *The ED-V protein barely suppresses MDA5-induced IFN-β promoter activity*

To explain the differential type I IFN-inducing abilities of ED versus wild-type strains, we transfected cDNAs encoding MV proteins into A549/CD150 cells, established in our laboratory (Tanabe et al., 2003). In these pilot studies, we found that expression of MV V protein suppresses IFN-β promoter activation, as reported by other groups (Nakatsu et al., 2008; Ohno et al., 2004; Takeuchi et al., 2003). We then focused on the function of the V and C proteins of various MV strains. The V and C proteins of MV are not essential products (Radecke and Billeter, 1996) but play important roles in MV virulence (Patterson et al., 2000). The V protein has been shown to inhibit IFN induction via binding to MDA5 (Childs et al., 2007, 2009). On the other hand, the C protein does not block the IFN-inducing pathway, but affects infectivity by acting as a regulator of viral RNA synthesis (Nakatsu et al., 2008). When A549/CD150 cells were stimulated with polyI:C or transfected with RIG-I or MDA5, efficient IFN-β promoter activation was detected using a reporter assay (Fig. 2A–C). Using this assay, we examined the effects of the transfected V and/or C proteins on IFN-β promoter activity. PolyI:C-induced IFN-β promoter activation was inhibited by the V protein expressed by wild-type strains, MS and IC-B, and an attenuated NV strain, which possesses DI RNA (Shingai et al., 2007). The ED-V pro-