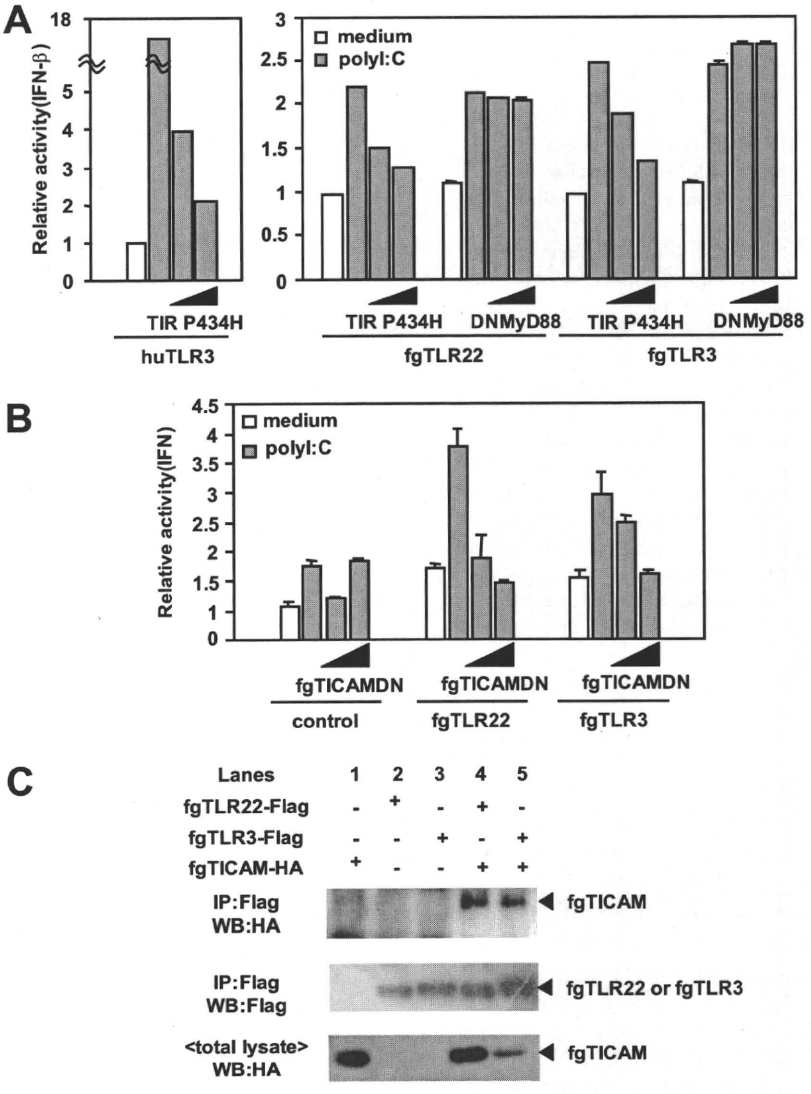

FIGURE 5. fg/huTICAM-1 is the adaptor for fgTLR22- and fgTLR3-mediated IFN promoter activation. *A*, Human (hu)TICAM-1 transmits signal for IFN-β promoter activation by fgTLR3 and fgTLR22 in HEK293 cells. HEK293 cells were transfected with fgTLR22 or fgTLR3 plasmid together with the plasmid encoding dominant-negative forms of huTICAM-1 or huMyD88. huTLR3 and TIR P434H were used as controls (*right panel*). Twenty-four hours later, cells were stimulated with poly(I:C) (10 μg/ml) for 6 h, and IFN-β promoter activation was determined by luciferase activity in the cell lysate. *B*, fgTICAM-1 acts as the adaptor for fgTLR3 and fgTLR22 to activate the fgIFN promoter in response to poly(I:C) in RTG-2 cells. RTG-2 cells were transfected with pEFBOS (fgTLR22), pEFBOS (fgTLR3), or pEFBOS together with dominant-negative forms of fgTICAM-1. After 24 h, cells were stimulated with poly(I:C) (10 μg/ml) for 6 h, and fgIFN promoter activity was determined by luciferase activity in cell lysate. *C*, fgTICAM-1 physically binds fgTLR3 and fgTLR22. HEK293 cells were transfected with plasmid with fgTLR22 or fgTLR3 together with plasmid of fgTICAM-1. Lysates from the cells transfected with the indicated vectors were immunoprecipitated (IP) with anti-Flag Ab and the samples were resolved on SDS-PAGE and analyzed by immunoblotting, which were probed with anti-HA Ab (*top panel*) or anti-Flag Ab (*middle panel*). fgTICAM-1 was detected in the blot of the total lysate by anti-HA Ab (*bottom panel*). Protein bands were developed by ECL kit. Arrows indicate HA-tagged fgTICAM-1 (*top and bottom panels*); Flag-tagged fgTLRs (*middle panel*).

than in control cells, and then gradually decreased in both types of cells (Fig. 3*B*).

Because human TLR3 expression is induced by poly(I:C) (32), we investigated whether teleost TLR22 or TLR3 transcriptions are activated by poly(I:C) stimulation. Endogenous rtTLR22 or rtTLR3 expression was scarcely observed without stimulation, and rtTLR22 expression was induced from 12 h after stimulation. rtTLR3 was also up-regulated but more mildly than fgTLR22 in response to poly(I:C) stimulation (Fig. 3*C*). These data are consistent with the notion that TLR22 is involved in the dsRNA recognition pathway.

Localization of fgTLR3 and fgTLR22

Why are there two TLRs that respond to poly(I:C) or dsRNA in teleosts? The answer may lie in the differences between the two TLRs. fgTLR3 and fgTLR22 preferentially recognize different sizes of dsRNA, as suggested by the reporter analyses. An additional difference is that fgTL22 localizes to the surface of the cell membrane while fgTLR3 localizes inside the cell. TLRs are type I transmembrane proteins, and their subcellular localizations are determined based on their primary structures and coupling proteins (33, 34). Human TLR1, 2, 4, 5, and 6 are expressed on the cell surface, but TLR3, 7, and 9 are mainly localized at intracellular compartments, endoplasmic reticulum (ER), and early endosomes (10, 35). In the case of human TLR3, its linker region between the transmembrane and TIR domain is a critical determinant for its localization (33). Human TLR3 and fgTLR3 share similar linker regions, but fgTLR22 does not have a similar linker sequence (Fig. 1*B*). Thus, we expected that fgTLR22 and fgTLR3 are distinctly localized.

To test this premise, we transfected HEK293 cells with myc-tagged fgTLR22 or Flag-tagged fgTLR3 and examined their expression using FACS analysis. fgTLR22 protein was observed partially on the cell surface (data not shown). On the cell surface, however, no fgTLR3 expression was observed, consistent with the case of dendritic cell human TLR3, which resides in the cytoplasmic compartments. Next, we overexpressed fgTLR22 or fgTLR3 in HeLa cells and visualized their localizations using a confocal microscope. YFP-labeled fgTLR3 was used in this study to avoid artificial deposition of fluorescence-labeled secondary Abs onto the cells. YFP labeling and HA or Flag labeling gave a similar imaging profile to fgTLR3 (data not shown). fgTLR3 was localized in the cytoplasm (Fig. 4). FgTLR3 was largely merged with calnexin (an integral protein localized in ER), suggesting that fgTLR3 localizes in the ER. In contrast, fgTLR22 was largely situated in close proximity to the cell surface, with only a minor population sitting in the cytoplasm (Fig. 4). These results confirm the predominant localization of fgTLR22 on the cell surface.

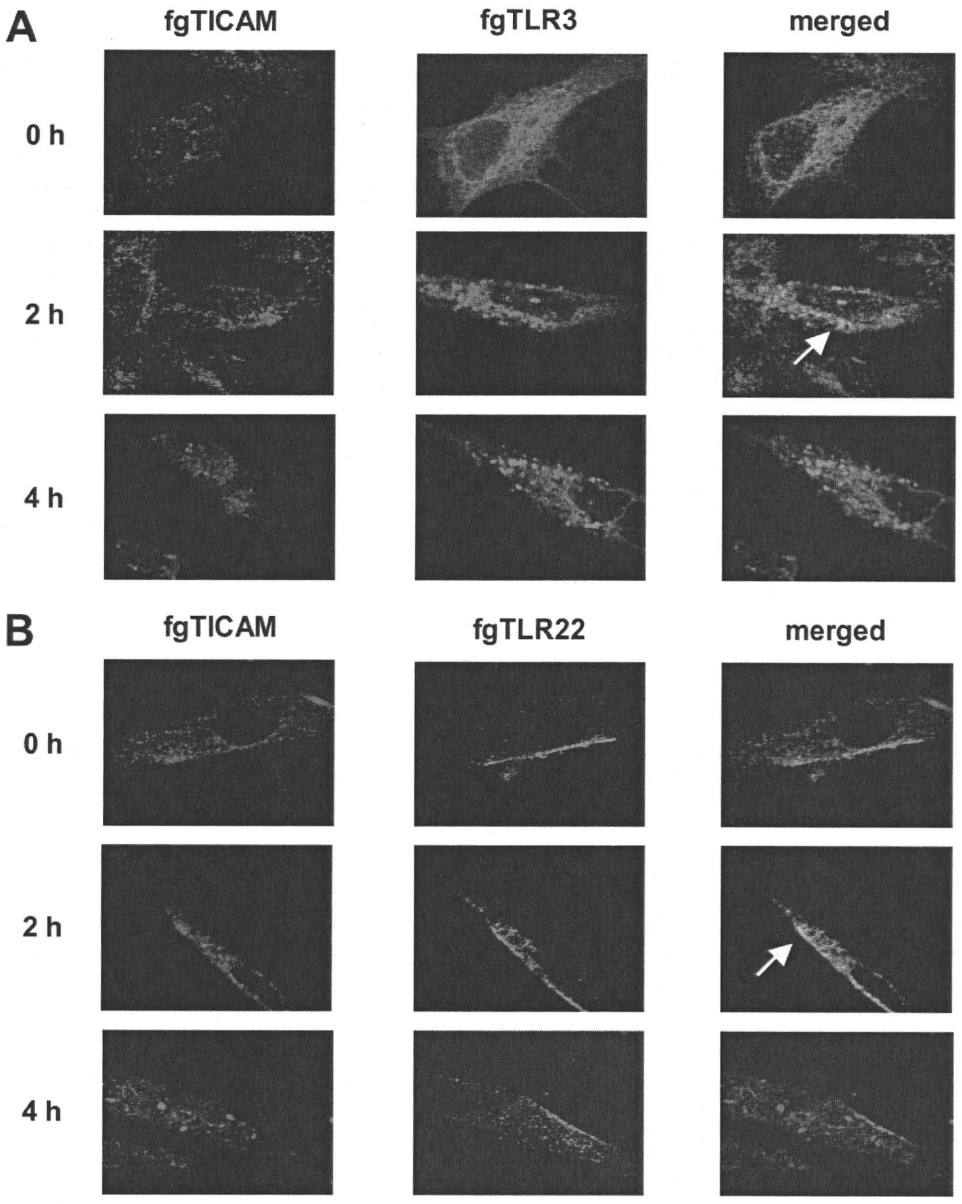

FIGURE 6. Dynamics of fgTICAM-1, fgTLR22, and fgTLR3 after poly(I:C) stimulation. RTG-2 cells onto coverslips were transfected with fgTLR22 (Flag-tagged) and fgTICAM-1 (HA-tagged) (*A*) or fgTLR3 (YFP) and fgTICAM-1 (HA-tagged) (*B*). Cells were allowed to stand for 24 h, stimulated with poly(I:C) (25 μg/ml) for the indicated intervals, and then stained with anti-Flag mAb and anti-HA pAb. Samples were analyzed by confocal microscopy. YFP and Flag labeling gave a similar localization profile to TLR3 (data not shown).

In the next experiment, we transfected OLHd-rRe3 fibroblastic cells derived from *O. latipes* (36) with fgTLR22 or fgTLR3 and tested their localizations. Although the transfection efficacy was very low in OLHd-rRe3 cells, we checked localization of TLRs by confocal analysis. As in the HeLa cells, cytoplasmic localization of fgTLR3 and cell surface-dominant localization of fgTLR22 were confirmed with OLHd-rRe3 cells (Fig. 4). Double staining of both fgTLR22 and fgTLR3 clearly supported their differential localizations. We also observed these localization differences in RTG-2 cells (data not shown). Taken together, fgTLR22 mainly localizes on the cell-surface membranes distinct from fgTLR3 in fish cells.

Adaptor selection of fgTLR22

Human TLR family proteins use four adaptor proteins, MyD88, Mal/Toll-IL-1R domain-containing adaptor protein (TIRAP), TICAM-1/TRIF, and TICAM-2/TRAM, to induce cytokine production (1, 2). Several teleost genomes encode three adaptor proteins, MyD88, TIRAP, and TICAM-1 (37). Teleost TICAM-2 has not been found and may have been lost in the teleost lineage during evolution (30). At first we used HEK293 cells to determine which human adaptor protein is compatible with fgTLR22 by reporter assay. FgTLR22 and a dominant-negative form of human MyD88 or TICAM-1 were transfected into HEK293 cells with the human IFN-β promoter reporter, and the cells were stimulated with poly(I:C). The dominant-negative form of human TICAM-1 (P434H) inhibited IFN-β promoter activation in fgTLR22-expressing cells, but the dominant-negative human MyD88 did not (Fig. 5*A*). Additionally, P434H inhibited fgTLR3-mediated IFN-β promoter activation in fgTLR3-expressing HEK293 cells (Fig. 5*A*). Thus, human TICAM-1 can act as an adaptor for fgTLRs.

Next, we made the dominant-negative form of fgTICAM-1, which has a mutation similar to that of the human dominant-negative form. We transfected fgTLR22, fgTLR3, and type I IFN reporter into RTG-2 cells with or without the dominant-negative form of fgTICAM-1 and then examined reporter activation. As in human cells, dominant-negative fgTICAM-1 inhibited the reporter activation in fgTLR22- and fgTLR3-transfected cells (Fig. 5*B*). Hence, fgTLR22 and fgTLR3 used fgTICAM-1 as the adaptor.

To further confirm the utilization of fgTICAM-1 as the adaptor by fgTLR22, we performed immunoprecipitation analyses. Full-length Flag-tagged fgTLR22 or fgTLR3 was transfected into HEK293 cells together with HA-tagged full-length fgTICAM-1.

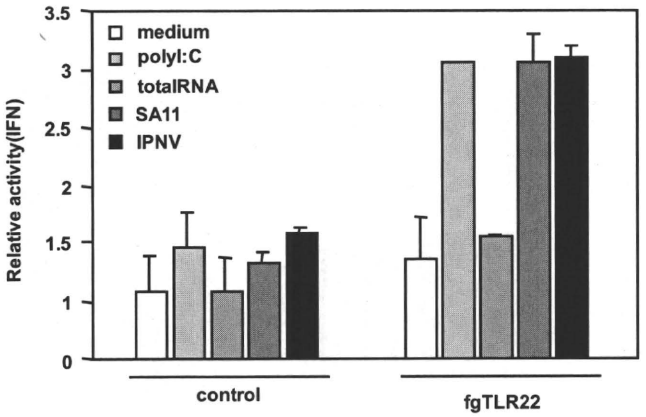

FIGURE 7. Viral dsRNA-mediated fgIFN induction interferes with fgTLR22. RTG-2 cells were transfected with the fgTLR22 plasmid (pEFBOS (fgTLR22)) or pEFBOS (vector only) and the fgIFN reporter plasmid. Twenty-four hours later, cells were treated for 6 h with medium only, poly(I:C), RTG-2 cell total RNA, or dsRNA of IPNV or rotavirus (SA11) origin. fgIFN activation was monitored by luciferase activity in the cell lysate.

We prepared cell lysates 24 h after transfection, and fgTLR22 and fgTLR3 were immunoprecipitated with anti-Flag Ab. Precipitates were analyzed by SDS-PAGE and stained with anti-Flag or HA Abs. Co-immunoprecipitation was confirmed with fgTICAM-1 and fgTLR22 or fgTLR3 (Fig. 5*C*), suggesting that fgTICAM-1 binds fgTLR22 and fgTLR3 in HEK cells.

Stimulation-induced recruitment of fgTICAM to fgTLR3 and fgTLR22

On cytological analyses with OLHd-rRe3 cells, overexpressed fgTICAM-1 was uniformly distributed over the cytoplasm without stimulation. The localization profile of fgTICAM-1was not much changed after poly(I:C) stimulation in later time points (Fig. 6). Likewise, a major population of fgTLR22 barely changed its localization even when stimulated with poly(I:C) (Fig. 6*B*). Only a small population appeared inside the cells in response to poly(I:C) and it partially merged with fgTICAM-1 (Fig. 6*B*). FgTLR3 was distributed in the cytoplasm in resting cells, but 2 h after the stimulation the fgTLR3 gathered to form "speckles" in the cytoplasm (Fig. 6*A*). Although some fgTLR22 and fgTLR3 colocalized with fgTICAM-1 on poly(I:C) stimulation, they gradually dissociated (Fig. 6). These data show that both fgTLR22 and fgTLR3 use TICAM-1 as the shuttling adaptor for IFN induction.

TLR22-mediated antiviral response in fish cells

Type I IFN is crucial for the antiviral response in mammals. Because fgTLR22 induced IFN in fish cells, we expected it to be required for cell protection against virus infection. To test this hypothesis, we prepared dsRNA of the IPNV (38, 39). IPNV genomic dsRNA was extracted from IPNV-infected RTG-2 cells. Isolated dsRNA of IPNV was poured over RTG-2 cells expressing fgTLR22. Like synthetic dsRNA or rotavirus-derived dsRNA (SA11), dsRNA of IPNV-induced endogenous IFN gene expression in fgTLR22-expressing cells compared with control vector-transfected cells (no induction of IFN) 2 h after stimulation (Fig. 7).

To test the resistance of RTG-2 cells to IPNV infection, we propagated a large-scale IPNV preparation amplified with RTG-2 cells. The preparation usually contained naked RNA of the IPNV genome. Thus, we prepared two infection sources for the assay: an IPNV preparation containing dsRNA, and an IPNV preparation depleted of free dsRNA. The latter sample was prepared by treating the IPNV preparation with RNase III (Fig. 8*A*). The IPNV preparations depleted of dsRNA infected RTG-2 cells and induced

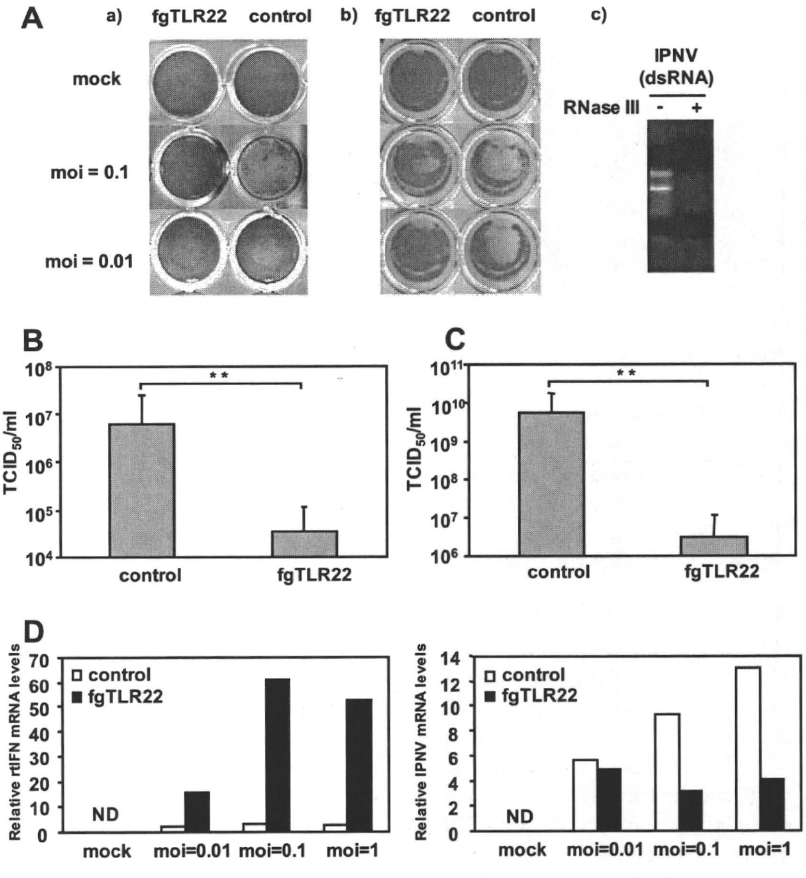

FIGURE 8. fgTLR22 expression protects cells from IPNV infection. *A*, RTG-2 cells were transfected with pEFBOS (fgTLR22) plasmid or empty vector, and 24 h later cells were exposed to the indicated moi of IPNV propagated with the RTG-2 cells. Intact IPNV (*a*) or a preparation pretreated with RNase III to remove contaminating dsRNA (*b*) was used as a virus source. dsRNA degradation was confirmed with agarose gel (*c*). At timed intervals (usually 7 days), when cells die by IPNV-induced cell death, CPE was observed under the microscope. Cells were fixed with 10% formaldehyde and stained with crystal violet. *B* and *C*, RTG-2 cells either transfected with mock or pEFBOS (fgTLR22) were infected with intact (*B*) or RNase-treated (*C*) IPNV (moi = 0.1). The medium was exchanged for removal of nonadherent viruses. Twenty-four hours after cells were replated, the supernatant was recovered and the titer checked by $TCID_{50}$. *D*, RTG-2 cells were transfected with pEFBOS (fgTLR22) or empty pEFBOS. Twenty-four hours later, cells were incubated with IPNV (moi = 0.01, 0.1, 1) for the indicated periods. After 24 h, the mRNA levels of rtIFN or IPNV were measured by quantitative PCR. Relative fold induction against the rtβ-actin level is shown. The experiments were performed three times and representative results are shown.

CPE followed by apoptosis (Fig. 8*A*). Infected RTG-2 cells were rescued from CPE if the cells expressed fgTLR22 beforehand (see the wells of moi of 0.01) (Fig. 8*A*).

We then quantitatively determined fgTLR22-mediated inhibition of IPNV infection. IPNV (moi = 0.1) (10 μg/ml) with (Fig. 8*B*) or without (Fig. 8*C*) naked dsRNA was added to TLR22-expressing RTG-2 cells. We measured the virus titer ($TCID_{50}$/ml) in the supernatants of control and fgTLR22-expressing cells. Twenty-four hours after infection, the IPNV titer was greatly decreased in fgTLR22-expressing cells compared with untransfected cells (Fig. 8, *B* and *C*). The IFN message was efficiently induced in cells with fgTLR22 concomitant with IPNV infection. Control cells, however, barely raise the IFN message in response to IPNV (Fig. 8*D*), suggesting that fgTLR22 has a critical role in IFN-mediated antiviral defense in fish cells. In conclusion, fgTLR22 governs antiviral response to a dsRNA virus IPNV via induction of fish IFN.

Discussion

Herein, we demonstrated that TLR22 is a dsRNA recognition receptor. Since fish possess both TLR3 and TLR22, they have a dual dsRNA recognition system. TLR3 and TLR22 recruit a common adaptor, TICAM-1. TLR22 preferentially recognizes long-sized dsRNA, localizes to the cell surface, and is widely distributed to tissue/organs (20). This is the first study to reveal that the TICAM-1 pathway serves as a key alert for an RNA virus sensor on the vertebrates.

According to bootstrap probability analysis, TLR22 does not belong to the TLR3 family; instead, it is proximal to mouse TLR13, which has not been characterized as a dsRNA-recognizing TLR. Thus, two arms of the TICAM-1 pathway have evolved as dsRNA receptors in fish, and only one (TLR3) has been preserved in mammals. Development of TLR22 instead of TLR3 may have some merit for protection against viruses with dsRNAs by augmenting the susceptibility of the local IFN response to long RNA duplexes in the vertebrates.

We wanted to clarify why teleosts need a cell surface RNA recognition system. Fish live in water and are exposed to many kinds of negative-stranded RNA viruses belonging to the Rhabdoviridae and to dsRNA viruses (40, 41). Bacteria such as *Rhodovulum sulfidophilum* and perhaps other species extracellularly liberate ribosomal and transfer RNAs (42). Thus, the sea may contain RNA viruses and RNA products of microbial origin. The sea is home to a unique and mysterious microbial environment. During evolution, vertebrates in water may have been protected from these pathogens by developing the set of RNA-sensing TLRs and the IFN system, which are distinct from those expressed in land animals. Our results indicate that RNA-sensing by TLRs protects fish from spreading or exacerbating infection. Viral RNAs often form a stem-loop or duplex signature (43) and are released from infected individual fish into the sea. TLR22 may sense such floating RNA as an infection threat.

Overexpressed teleost TLR22 protects host cells from infection with IPNV, which is a naked bisegmented dsRNA virus belonging to the family Birnaviridae (44). Birnaviruses have a single T = 13 icosahedral shell composed of 120 subunits and they lack the characteristic inner capsid. Aquatic birnaviruses are distributed worldwide, can infect a range of fish and shellfish species (44, 45), and are viral pathogens that cause problems in fry and young fish. Although teleosts have the gene encoding a putative ortholog of the cytoplasmic RNA sensor MDA5 (24), IPNV efficiently infect teleost cells unless TLR22 is expressed in some population of cells. Thus, fish MDA5 is insufficient for protection against this type of dsRNA virus. Although not all cells express TLR22, IFN seems to be sufficiently induced by TLR22-expressing cells (Fig. 8*D*) to provide antiviral environment in surrounding cells, resulting in host cell protection. However, how TLR22 detects the IPNV infection remains to be clarified. The necessity of TLR22 and the mode of its dsRNA recognition in fish are of interest for further investigation.

A notable difference between fgTLR22 and surface-expressed human TLR3 is that fgTLR22 is ubiquitous whereas surface TLR3 is expressed exclusively together with endosomal/ER TLR3 in TLR3-positive cells. In humans, cell-surface TLR3 is therefore distributed limitedly to the fibroblasts and epithelial cells. We previously found human TLR3 on the cell surface of fibroblasts, which therefore binds anti-TLR3 mAb, TLR3.7 (46). TLR3 on the fibroblasts recognizes exogenously added poly(I:C) to confer IFN-β induction (46). The IFN-inducing properties of human TLR3 by fibroblasts are blocked by this mAb. The result is a unique and exceptional TLR3 feature that reflects a differential TLR3 function (10). Ultimately, in humans TLR3 is expressed in the cytoplasmic compartments and on the surface of fibroblasts (33). Other reports also found that human bronchial, bile duct, and intestinal epithelial cells express TLR3 on the cell-surface membrane (47–50). In this view, surface-expressed human TLR3 is a functional remnant of fish TLR22: TLR3 functions in the mucosal region wherein body fluids are continuously in contact with the flora. Because the cell surface-associated dsRNA recognition is important even in humans, TLR3 is expressed on human fibroblasts and epithelial cells (50–52).

In this study, we found that fgTLR3 and fgTLR22 recognize its ligand dsRNA at the different sites and recruit fgTICAM-1. Although TLR22 expression is limited to part of the cell surface, a colocalization study with cholera toxin did not support its expression being restricted to lipid rafts (data not shown). Unlike the case of human TLR3, the chloroquin treatment of HEK293 cells expressing TLR22 did not abrogate the activation of the IFN-β reporter gene by poly(I:C) (data not shown). Thus, it is unlikely that TLR22 captures poly(I:C) at the endosome, as does TLR3. Human and mouse TLR3 in myeloid dendritic cells have a role in driving the cells to a maturation stage sufficiently activating NK and T cells (9, 53). It is intriguing whether TLR22 possesses this function in dendritic cells.

In this context, the question is how TLR22 assembles TICAM-1 to transmit the dsRNA-recognition signal. Possible answers may lie in the fish-specific TLR22 pathway and in the functional difference between mammalian and teleost TICAM-1. A recent study of zfTICAM-1 suggested that overexpression of zfTICAM-1 activates the zfIFN promoter but that TICAM-1 does not interact with zfTRAF6 (30). The zfTICAM-1 N-terminal region does not contain a TRAF6-binding motif, and the C-terminal region of zfTICAM-1 sufficiently activates the IFN promoter, suggesting the involvement of RIP1-mediated NF-κB activation in zfIFN promoter activation (30). In OLHd-rRe3 cells, a zebrafish minimal Mx promoter that contains two ISRE barely responds to overexpressed fgTICAM-1 or its N-terminal-deleted form, but it enhances activation of the zfIFN promoter, as shown by reporter assay. In human TICAM-1, the N-terminal region is mandatory for IRF-3-mediated type I IFN induction in the human system (14, 54). Ultimately, fish TICAM-1 behaves like human TICAM-1, although fish TICAM-1 does not mediate IRF-3 for activation of the IFN-β promoter (30). It takes a longer period for fish cells (compared with human cells; see Refs. 54, 55) to evoke TLR-TICAM-1 interaction in response to poly(I:C), which may be attributable to the temperature (~20°C) where the fish cells are grown, rather than to the difference of signaling mode. Hence, the TICAM-1 pathway is conserved across fish and humans, but its molecular

bases for IFN induction are different between them. Our speculation is that fish cells have an IFN output similar to that of human cells (54, 55) but that the signal cascade for IFN production is modally different. Teleost TICAM-1, which is partly dissimilar to human TICAM-1 in its structure, might explain the differential selection of their signal pathways.

An alternative possibility is that the IFN-inducing capacity due to the recruitment of TICAM-1 lies within the molecular feature of TLR22. Supporting evidence is that surface-expressed TLR22 retains the capacity to confer the responsiveness to poly(I:C) not only in fish RTG-2 cells but also in human HEK293 cells. FgTLR22 and even only the TIR domain of fgTLR22 can couple with human TICAM-1 as well as fish TICAM-1 to activate the human and fish ISRE promoter. Either way, TLR3 is not the only partner of TICAM-1 in the water vertebrates.

Acknowledgments

We are grateful to Drs. A. Ishii, M. Shingai, M. Sasai, K. Funami, and T. Ebihara in our laboratory for their critical discussions. Thanks are also due to Drs. T. Fujita (Kyoto Univiversity, Kyoto), T. Taniguchi (Tokyo Univiversity, Tokyo), and T. Maniatis (Harvard University, Boston, MA) for providing their plasmids.

Disclosures

The authors have no financial conflicts of interest.

References

1. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783–797.
2. Honda, K., A. Takaoka, and T. Taniguchi. 2006. Type I interferon gene induction by the interferon regulatory factor family of transcription factors. *Immunity* 25: 349–360.
3. Samuel, C. E. 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev.* 14: 778–809.
4. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5: 987–995.
5. Yoneyama, M., and T. Fujita. 2007. Function of RIG-I-like receptors in antiviral innate immunity. *J. Biol. Chem.* 28: 15315–15318.
6. Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, et al. 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science* 314: 994–997.
7. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* 314: 997–1001.
8. Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, et al. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441: 101–105.
9. Matsumoto, M., and T. Seya. 2008. TLR3 signaling inducing IFN in response to dsRNA and polyI:C. *Adv. Drug Delivery Rev.* 60: 805–812.
10. Matsumoto, M., K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, and T. Seya. 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* 171: 3154–3162.
11. Oganesyan, G., S. K. Saha, B. Guo, J. Q. He, A. Shahangia, B. Zarnegar, A. Perry, and G. Cheng. 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and-independent antiviral response. *Nature* 439: 208–211.
12. Hacker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L. C. Hsu, G. G. Wang, M. P. Kamps, E. Raz, H. Wagner, G. Hacker, et al. 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 439: 204–207.
13. Ryzhakov, G., and F. Randow. 2007. SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. *EMBO J.* 26: 3180–3190.
14. Sasai, M., H. Oshiumi, M. Matsumoto, N. Inoue, F. Fujita, M. Nakanishi, and T. Seya. 2005. Cutting edge: NF-κB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *J. Immunol.* 174: 27–30.
15. Sasai, M., M. Shingai, K. Funami, M. Yoneyama, T. Fujita, M. Matsumoto, and T. Seya. 2006. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. *J. Immunol.* 177: 8676–8683.
16. Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, et al. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424: 743–748.
17. Stack, J., I. R. Haga, M. Schroder, N. W. Bartlett, G. Maloney, P. C. Reading, K. A. Fitzgerald, G. L. Smith, and A. G. Bowie. 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *J. Exp. Med.* 201: 1007–1018.
18. Altmann, S. M., M. T. Mellon, D. L. Distel, and C. H. Kim. 2003. Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. *J. Virol.* 77: 1992–2002.
19. Robertsen, B. 2006. The interferon system of teleost fish. *Fish Shellfish Immunol.* 20: 172–191.
20. Oshiumi, H., T. Tsujita, K. Shida, M. Matsumoto, K. Ikeo, and T. Seya. 2003. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. *Immunogenetics* 54: 791–800.
21. Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem. 2005. The evolution of vertebrate Toll-like receptors. *Proc. Natl. Acad. Sci. USA* 102: 9577–9582.
22. Meijer, A. H., S. F. Gabby Krens, I. A. Medina Rodriguez, S. He, W. Bitter, B. Ewa Snaar-Jagalska, and H. P. Spaink. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. *Mol. Immunol.* 40: 773–783.
23. Jault, C., L. Pichon, and J. Chluba. 2004. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. *Mol. Immunol.* 40: 759–771.
24. Ishii, A., M. Kawasaki, M. Matsumoto, S. Tochinai, and T. Seya. 2007. Phylogenetic and expression analysis of amphibian *Xenopus* Toll-like receptors. *Immunogenetics* 59: 281–293.
25. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adapter molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nat. Immunol.* 4: 161–167.
26. Tsujita, T., H. Tsukada, M. Nakao, H. Oshiumi, M. Matsumoto, and T. Seya. 2004. Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*). *J. Biol. Chem.* 279: 48588–48597.
27. Higuchi, M., A. Matsuo, M. Shingai, K. Shida, A. Ishii, K. Funami, Y. Suzuki, H. Oshiumi, M. Matsumoto, and T. Seya. 2007. Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily. *Dev. Comp. Immunol.* 32: 147–155.
28. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388: 394–397.
29. Okahira, S., F. Nishikawa, S. Nishikawa, T. Akazawa, T. Seya, and M. Matsumoto. 2005. Interferon-β induction through Toll-like receptor 3 depends on double-stranded RNA structure. *DNA Cell Biol.* 24: 614–623.
30. Sullivan, C., J. H. Postlethwait, C. R. Lage, P. J. Millard, and C. H. Kim. 2007. Evidence for evolving Toll-IL-1 receptor-containing adaptor molecule function in vertebrates. *J. Immunol.* 178: 4517–4527.
31. Jensen, I., A. Albuquerque, A. I. Sommer, and B. Robertsen. 2002. Effect of polyI:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. *Fish Shellfish Immunol.* 13: 311–326.
32. Carty, M., R. Goodbody, M. Schroder, J. Stack, P. N. Moynagh, and A. G. Bowie. 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat. Immunol.* 7: 1074–1081.
33. Funami, K., M. Matsumoto, H. Oshiumi, T. Akazawa, A. Yamamoto, and T. Seya. 2004. The cytoplasmic "linker region" in Toll-like receptor 3 controls receptor localization and signaling. *Int. Immunol.* 16: 1143–1154.
34. Nishiya, T., E. Kajita, S. Miwa, and A. L. Defranco. 2005. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *J. Biol. Chem.* 280: 37107–37117.
35. Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* 5: 190–198.
36. Hirayama, M., H. Mitani, and S. Watabe. 2006. Temperature-dependent growth rates and gene expression patterns of various medaka *Oryzias latipes* cell lines derived from different populations. *J. Comp. Physiol. B* 176: 311–320.
37. Purcell, M. K., K. D. Smith, L. Hood, J. R. Winton, and J. C. Roach. 2006. Conservation of Toll-like receptor signaling pathways in teleost fish. *Comp. Biochem. Physiol. D Genomics Proteomics* 1: 77–88.
38. Jensen, V., and B. Robertsen. 2000. Cloning of an Mx cDNA from Atlantic halibut (*Hippoglossus hippoglossus*) and characterization of Mx mRNA expression in response to double-stranded RNA or infectious pancreatic necrosis virus. *J. Interferon Cytokine Res.* 20: 701–710.
39. Collet, B., E. S. Munro, S. Gahlawat, F. Acosta, J. Garcia, C. Roemelt, J. Zou, C. J. Secombes, and A. E. Ellis. 2007. Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. *Fish Shellfish Immunol.* 22: 44–56.
40. Phelan, P. E., M. E. Pressley, P. E. Witten, M. T. Mellon, S. Blake, and C. H. Kim. 2005. Characterization of snakehead rhabdovirus infection in zebrafish (*Danio rerio*). *J. Virol.* 79: 1842–1852.
41. Nishizawa, T, S. Kinoshita, and M. Yoshimizu. 2005. An approach for genogrouping of Japanese isolates of aquabirnaviruses in a new genogroup, VII, based on the VP2/NS junction region. *J. Gen. Virol.* 86: 1973–1978.
42. Ando, T., H. Suzuki, S. Nishimura, T. Tanaka, A. Hiraishi, and K. Kikuchi. 2006. Characterization of extracellular RNAs produced by the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. *J. Biochem.* 139: 805–811.
43. Shingai, M., T. Ebihara, N. A. Begum, A. Kato, T. Honma, K. Matsumoto, H. Saito, H. Ogura, M. Matsumoto, and T Seya. 2007. Differential type I interferon (IFN) inducing abilities of wild-type vs. vaccine strains of measles virus. *J. Immunol.* 179: 6123–6133.
44. Coulibaly, F., C. Chevalier, I. Gutsche, J. Pous, J. Navaza, S. Bressanelli, B. Delmas, and F. A. Rey. 2005. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. *Cell* 120: 761–772.
45. Jensen, I., and B. Robertsen. 2002. Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia

virus and infectious pancreatic necrosis virus. *Fish Shellfish Immunol.* 13: 221–241.
46. Matsumoto, M., S. Kikkawa, M. Kohase, K. Miyake, and T. Seya. 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem. Biophys. Res. Commun.* 293: 1364–1369.
47. Rudd, B. D., J. J. Smit, R. A. Flavell, L. Alexopoulou, M. A. Schaller, A. Gruber, A. A. Berlin, and N. W. Lukacs. 2006. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. *J. Immunol.* 176: 1937–1942.
48. Liu, P., M. Jamaluddin, K. Li, R. P. Garofalo, A. Casola, and A. R. Brasier. 2007. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J. Virol.* 81: 1401–1411.
49. Cario, E., and D. K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect. Immun.* 68: 7010–7017.
50. Nakamura, M., K. Funami, A. Komori, T. Yokoyama, Y. Aiba, A. Araki, Y. Takii, M. Ito, M. Matsuyama, M. Koyabu, et al. 2008. Increased expression of TLR3 in human intrahepatic biliary epithelial cells at the site of ductular reaction in primary billiary cirrhosis. *Hepatol. Intern.* In press.
51. Harada, K., Y. Sato, K. Itatsu, K. Isse, H. Ikeda, M. Yasoshima, Y. Zen, A. Matsui, and Y. Nakanuma. 2007. Innate immune response to double-stranded RNA in biliary epithelial cells is associated with the pathogenesis of biliary atresia. *Hepatology* 46: 1146–1154.
52. Zhou, R., H. Wei, R. Sun, and Z. Tian. 2007. Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice. *J. Immunol.* 178: 4548–4556.
53. Ebihara, T., M. Shingai, M. Matsumoto, K. Shimotohno, T. Wakita, and T. Seya. 2008. Hepatitis C virus (HCV)-infected apoptotic cells extrinsically modulate dendritic cell function to activate T cells and NK cells. *Hepatology.* 48: 48–58.
54. Funami, K., M. Sasai, Y. Ohba, H. Oshiumi, T. Seya, and M. Matsumoto. 2007. Spatiotemporal mobilization of TICAM-1 in response to dsRNA. *J. Immunol.* 179: 6827–6830.
55. Funami, K., M. Sasai, H. Oshiumi, T. Seya, and M. Matsumoto. 2008. Homo-oligomerization is essential for Toll/IL-1 receptor domain containing adaptor molecule-1 signaling. *J. Biol. Chem.* 283: 18283–18291.

International Immunology, Vol. 20, No. 9, pp. 1169–1180
doi:10.1093/intimm/dxn074

Soluble G protein of respiratory syncytial virus inhibits Toll-like receptor 3/4-mediated IFN-beta induction

Masashi Shingai[1,4], Masahiro Azuma[1], Takashi Ebihara[1], Miwa Sasai[1,5], Kenji Funami[1,6], Minoru Ayata[2], Hisashi Ogura[2], Hiroyuki Tsutsumi[3], Misako Matsumoto[1] and **Tsukasa Seya[1]**

[1]Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan
[2]Department of Virology, Osaka City University, Asahimachi 1-4-3, Abeno-ku, Osaka 545-8585, Japan
[3]Department of Pediatrics, Sapporo Medical University, School of Medicine, Minami-1, Nishi-16, Chuoh-ku, Sapporo 060-8543, Japan

[4]Present address: Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA
[5]Present address: Section of Immunobiology, Yale University School of Medicine, 300 Cedar Street, TAC S640, New Haven, CT 06520, USA
[6]Present address: Medical Research Center, Keio University, Shinanomachi 35, Shinjuku-ku, Tokyo 160-8582, Japan

Keywords: dendritic cells, respiratory syncytial virus, TICAM-1 (TRIF), Toll-like receptor, type I IFNs

Abstract

Monocyte-derived dendritic cells (mDCs) recognize viral RNA extrinsically by Toll-like receptor (TLR) 3 on the membrane and intrinsically retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5) in the cytoplasm to induce type I IFNs and mDC maturation. When mDCs were treated with live or UV-irradiated respiratory syncytial virus (RSV), early (~4 h) induction of IFN-β usually occurs in other virus infections was barely observed. Live RSV subsequently replicated to activate the cytoplasmic IFN-inducing pathway leading to robust type I IFN induction. We found that RSV initial attachment to cells blocked polyI:C-mediated IFN-β induction, and this early IFN-β-modulating event was abrogated by antibodies against envelope proteins of RSV, demonstrating the presence of a IFN-regulatory mode by early RSV attachment to host cells. By IFN-stimulated response element (ISRE) reporter analysis in HEK293 cells, polyI:C- or LPS-mediated ISRE activation was dose dependently inhibited by live and inactive RSV to a similar extent. Of the RSV envelope proteins, simultaneously expressed or exogenously added RSV G or soluble G (sG) proteins inhibited TLR3/4-mediated ISRE activation in HEK293 cells. sG proteins expressed in cells did not affect the RIG-I/MDA5 pathway but inhibited the TLR adaptor TRIF/TICAM-1 pathway for ISRE activation. Finally, extrinsically added sG protein suppressed the production of IFN-β in mDCs. Although the molecular mechanism of this extrinsic functional mode of the RSV G glycoprotein (G protein) remains undetermined, G proteins may neutralize the fusion glycoprotein function that promotes IFN-mediated mDC modulation via TLR4 and may cause insufficient raising cell-mediated immunity against RSV.

Introduction

Respiratory syncytial virus (RSV) is a member of the Paramyxoviridae family consisting of a negative-strand RNA genome in a nucleocapsid. RSV preferentially infects airway epithelial cells, causing bronchiolitis and respiratory infections (1) and can exacerbate asthma and chronic obstructive pulmonary diseases (1). However, an effective vaccine for RSV is not yet available. Recurrent RSV infections are often observed in humans, and this is due to the failure of the hosts to raise long-lasting immunity against RSV (1). Recent reports suggested that cell-mediated immunity, including CTLs, NK and B cells, develops followed by maturation of monocyte-derived dendritic cells (mDCs) (2). These

Correspondence to: T. Seya; E-mail: seya-tu@pop.med.hokudai.ac.jp

Received 20 September 2007, *accepted* 9 June 2008
Transmitting editor: T. Takai
Advance Access publication 8 July 2008

lymphocytes produce IFN-γ which orchestrates the acquired immune response to eradicate viral infection (3). Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors and NOD-like receptors are expressed in dendritic cells (DCs) and play a major role in driving the lymphocyte-mediated immune responses (4). Possible involvement of TLR3 and its response in RSV infectious signs has been reported (5–7), although how RSV induces host immune modulation via the TLR3 remains largely unclear.

Type I IFNs serve as antiviral factors. Several reports have suggested the involvement of TLR3 (5, 7) and RIG-I (6) in RSV-mediated IFN-α/β induction and cellular responses. RIG-I preferentially recognizes 5′-triphosphate RNA (8, 9) in addition to double-stranded (ds)RNA, whereas TLR3 captures only dsRNA. Their signaling pathways partially overlap in that they converge upon the IFN-regulatory factor (IRF)-3-activating kinase complex for activation of the IFN-β promoter (10). Bronchial epithelial cells and mDCs preserve these receptors and downstream signaling pathways. mDC TLR3 particularly plays a crucial role in driving mDCs to direct CTL- and NK-inducing maturation as well as RSV infection-mediated type I IFN production (11, 12).

For induction of type I IFNs and NK/CTL activation, the cytoplasmic Toll–IL-1R (TIR) homology domain of TLR3 recruits the adaptor molecule TICAM-1 (TRIF) (13, 14), while LPS allows TLR4 to recruit the adaptor molecules TICAM-2 (TRAM) and TICAM-1 (15, 16). Thus, TICAM-1 is the common adaptor in the pathways of TLR3 and TLR4. Both pathways activate IRF-3 and IRF-7 through a MyD88-independent pathway, resulting in IFN-β production. Extrinsic supplement of viral dsRNA can activate the TICAM-1 pathway (17). On the other hand, RIG-I and melanoma differentiation-associated gene 5 (MDA5) reside in the cytoplasm and interact with a mitochondrial protein, IFN-β promoter stimulator 1 (IPS-1)/mitochondria antiviral signaling (MAVS)/VISA/Cardif, to activate IRF-3 and IRF-7 (18–21). Only intrinsically produced viral RNA is a ligand for the cytoplasmic IFN-inducing sensors. Studies on how these pathways evoke mDC-mediated cellular immunity are in progress with special interest (22). Although there is a MyD88-dependent pathway for IFN induction in plasmacytoid DCs (23–25), this pathway does not function in mDCs. Accordingly, we focus on the role of the TICAM-1 and IPS-1 pathways in RSV-mediated mDC functional modulation.

In the virus side, what RSV factors are associated with modulation of mDC maturation remain largely unknown. In cytoplasmic RSV proteins, the NS1 and NS2 proteins are shown to antagonize IFN response (26, 27). Nevertheless, type I IFN is induced in RSV-replicating cells although the amounts of IFN are relatively low. The envelope of RSV contains three transmembrane surface proteins, the fusion glycoprotein (F protein), G glycoprotein (G protein) and SH protein. F protein is responsible for fusion of the viral envelope with the plasma membrane of the host target cell (28). The F protein may induce activation of nuclear factor-κB (NF-κB) and the IFN-β promoter via TLR4 (29, 30). In addition, the F protein of RSV serves as an agonist of TLR4 and induces pro-inflammatory cytokines (29). On the other hand, the G protein, which mediates attachment of the virus particle to the target cell (31), and SH protein are not functionally well understood (32). Infected cells also produce a smaller secreted form of the G protein [soluble G (sG) protein] besides the transmembrane type G protein (33). The RSV G protein has been implicated in altered cytokine and chemokine expression by pulmonary leukocytes (34). Yet, there has been no report on the RSV surface proteins that affect cytoplasmic IFN-inducing events. Accordingly, no report has mentioned the possible association between the RSV G/SH proteins and the TLR pathways in RSV infection.

Here, we discovered a role of the RSV G protein in mDC IFN response. This protein inhibits the TLR3/4-mediated IFN-β promoter activation through RSV–host cell interaction. A possible target for the G protein attachment to cells is the TICAM-1 pathway, thereby TLR3/4-mediated type I IFN induction being prohibited. The RSV G protein may act as a buffer for evoking cell-mediated TLR3/4-derived immunity. Possible roles for the function of the G protein in the RSV infection are also discussed.

Methods

Cell culture, viruses and reagents

HEp-2, Vero and HEK293 cells were maintained in DMEM (Invitrogen, San Diego, CA, USA) supplemented with 10% heat-inactivated FCS (JRH Biosciences, Lenexa, KS, USA) and antibiotics. Human RSV field-isolate strain (RSV2177) in subgroup B was isolated and propagated with HEp-2 cells. The accession numbers of *NS1*, *NS2*, *N*, *G*, *F* and *SH* genes were AB245473–AB245478. The titer of RSV2177 was determined by 50% tissue culture infective dose with HEp-2 cells. Measles virus (MV) Edmonston strain was passaged and titrated in Vero cells. RSV and MV were inactivated by UV irradiation at 1.5 J cm^{-2}. Poly I:C was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Polymyxin B, LPS from *Escherichia coli* serotype 0111:B4, was from Sigma Chemical Co., St Louis, MO, USA. The mycoplasma lipopeptide macrophage-activating lipopeptide-2 (MALP-2) was prepared as described (35). MALP-2 and poly I:C were treated with polymyxin B (10 μg ml^{-1}) (an LPS inhibitor) for 1 h at 37°C before stimulation of cells (35). Usually, 50 or 10 μg ml^{-1} of poly I:C, 100 ng ml^{-1} of LPS and 100 nM of MALP-2 were utilized for TLR stimulation. Mouse IgG, mouse IgG2b and anti-Flag M2 mAb and anti-Flag polyclonal antibodies were obtained from Sigma; anti-CD80 and anti-HLA-DR mAbs were obtained from Immunotech (Marseille, France); anti-CD83 mAb was obtained from Cosmo Bio (Tokyo, Japan); anti-CD86 mAb was obtained from Ancell (Bayport, MN, USA); anti-CD40 mAb was from PharMingen (San Diego, CA, USA); FITC-conjugated goat anti-mouse and anti-rabbit IgG $F(ab')_2$ and HRP-conjugated goat anti-rabbit Igs were obtained from American Qualex Manufacturers (Bayport San Clemente, CA, USA) and FITC-labeled and non-labeled goat anti-RSV polyclonal antibody was from Chemicon.

Preparation of DCs (mDCs)

Human PBMCs were isolated from buffy coat of normal healthy donors by methylcellulose sedimentation followed by standard density-gradient centrifugation with Ficoll–Hypaque (Amersham Biosciences, Piscataway, NJ, USA) (35). For human immature DC preparation, CD14-positive monocytes

were prepared from huPBMC by using MACS system (Miltenyi Biotec, Gladbach, Germany) with anti-human CD14 mAb-conjugated microbeads and kept in RPMI-1640 (Invitrogen) containing 10% FCS, 500 IU ml^{-1} human granulocyte macrophage colony-stimulating factor, 100 IU ml^{-1} human IL-4 (Pepro Tech, London, UK) and antibiotics for 6 days. Morphological changes were examined by phase contrast microscopy (Olympus IX-70, Tokyo, Japan).

FACS cytometric analysis of cell-surface antigens

FACS methods were described previously (35). Briefly, cells were suspended in PBS containing 0.1% sodium azide and 1% BSA (FACS buffer) and incubated for 30 min at 4°C with relevant or control mAbs, followed by FITC-labeled anti-mouse IgG $F(ab')_2$. In some experiments, cells were directly stained with FITC-labeled anti-RSV polyclonal antibody. Cells were washed, and their fluorescence intensities were measured by FACS.

Determination of human tumor necrosis factor-α and IFN-β level

Quantitative PCR and ELISA were used for this purpose. Culture media were centrifuged to remove cell debris and the supernatants were stored at −80°C until the assay. The level of secreted human tumor necrosis factor-α (TNF-α) or IFN-β in the culture medium was determined with ELISA kits (Amersham Pharmacia and FUJIREBIO, Tokyo, Japan). The detection limits of human TNF-α and IFN-β were <5 pg ml^{-1} and <2.5 U ml^{-1}, respectively. Quantitative PCR and the primers for this assay were performed as described previously (36).

RSV sequences and plasmid construction

Total RNA from RSV2177-infected HEp-2 cells was extracted with RNeasy mini kit (Qiagen). After DNase treating, 1 μg of total RNA was incubated at 70°C for 5 min, kept on ice for 2 min and reverse transcription was performed with MMLV–reverse transcriptase (Promega, Madison, WI, USA) at 37°C for 90 min followed by PCR. Detection of RSV subgroup was performed by PCR with subgroup-specific primer sets (37) (RSV/SH A 5′-TCGAGTCAACACATAGCATTC-3′ and RSV/F1 5′-CAACTCCATTGTTATTTGCC-3′ for RSV subgroup A and RSV/SH B 5′-CATAGTATTCTACCATTATGC-3′ and RSV/F1 for RSV subgroup B). Direct sequences were detected from the amplified PCR fragments with conserved sequence primer sets among RSVs (RSV/Fm01 5′-GGCAAATAACAATGGAGYTGC-3′ and RSV/Fg01 5′-TTGTWRRAACATGATYAGGTG-3′ for F gene, RSV/Gm01 5′-GGCAAATGCAACCATGTCCAA-3′ and RSV/Gg01 5′-ACCCAATCACATGCTTAGTTATTC-3′ for G gene, RSV/SHm01 5′-ATGGGAAATACATCCAT-3′ and RSV/SHg01 5′-CACAGCATAATGGTAGA-3′ for SH gene and RSV/NPm01 5′-ATGGCTCTTAGCAAAG-3′ and RSV/NPg01 5′-TTAAAGCTCTACATCAT-3′ for NP gene). The nucleotide sequences of these PCR fragments were confirmed by direct sequencing. The consensus sequences obtained from the amplicons were inserted into a plasmid vector (pEFBos or $pCXN_2$), and the clones were modified by addition of Flag-tag, exchanging of signal sequence and/or truncation of the cytoplasmic and transmembrane regions.

Plasmid transfection and luciferase assay

A luciferase reporter plasmid, pISRE-Luc, was from Stratagene (Stratagene, La Jolla, CA, USA) and pELAM-Luc reporter plasmid was constructed as referred in Kurt-Jones *et al.* (29). pRL-TK vector was from Promega. A plasmid for human TLR3 and TICAM-1 expression was described previously (13). Plasmids for human TLR4, MD-2 and CD14 expression were kindly provided by K. Miyake (The University of Tokyo, Tokyo), TANK-binding kinase 1 (TBK1) expression by M. Nakanishi (The Nagoya City University, Nagoya) and IκB kinase-related kinase ε (IKKε) expression by T. Maniatis (Harvard Medical School, Boston, MA, USA). Plasmids for constitutive active forms of RIG-I and MDA5 (ΔRIG-I and MDA5N) expression were kindly provided by T. Fujita (The University of Kyoto, Kyoto). All transfection was carried out on HEK293 cells growing on 24-well plates. Usually, 100 ng of TLR3/pEFBos or TLR4/pEFBos, 100 ng of MD-2/pEFBos, 100 ng of CD14/pEFBos, 100 ng of luciferase reporter gene plasmid (firefly luciferase, experimental reporter) and 3 ng of pRL-TK vector (Renilla luciferase for internal control) were introduced into cells by LipofectAMINE 2000 (Invitrogen) according to the manufacture's procedure. At 24 h post-transfection, cells were stimulated with various stimulators for 6 h. Cells were then harvested with trypsin, washed with PBS and treated with 20 μl of Passive Lysis Buffer (Promega). After 6-h incubation, cells were lysed with lysis buffer and the assay was performed using dual-luciferase reporter assay system. Fold induction against the control medium is indicated.

Immunoprecipitation, SDS–PAGE and western blotting

Cells were washed in PBS (pH 7.4) and solubilized with 100 μl of 1% (v/v) Triton X-100 containing 137 mM NaCl, 2 mM EDTA and 1 mM phenylmethylsulfonylfluoride. After centrifugation (10 000 × *g* for 10 min), proteins in cell lysate or culture supernatant were immunoprecipitated with anti-Flag mAb. Immunoprecipitants were washed and eluted with Flag peptide. The eluted samples were heated or non-heated and were subjected to SDS–PAGE under reducing or non-reducing conditions. Proteins were transferred onto nylon membranes. The membranes were incubated with 10% skimmed milk containing 5% goat serum for 30 min at room temperature, followed by the addition of anti-Flag pAbs. One hour later, the membranes were washed extensively with PBS containing 0.5% Tween 20 and then incubated with 5 μg of HRP-conjugated goat anti-rabbit IgG antibody for 1 h at 37°C. Following second incubation, the membranes were washed with PBS–Tween 20 and proteins were detected with an ECL chemiluminescence kit (Amersham Biosciences).

Endoglycosidase digestion

Protein samples were made up to a final concentration of either 100 mM Tris–HCl (pH 8.6), 0.1% SDS and 1% NP-40 or 50 mM sodium citrate (pH 5.0) and 0.5% SDS and incubated at 37°C for 14 h with endoglycosidase F (Takara) or endoglycosidase H (Seikagaku Corporation, Tokyo, Japan), respectively, as previously reported (38). The samples were analyzed on SDS–PAGE under reducing and non-reducing conditions.

RSV treatment of human cells

Human cells (mDCs and HEK293 cells) were transfected with pGV-E2/huELAM (ELAM promoter-linked firefly luciferase) or pISRE-Luc [IFN-stimulated response element (ISRE) promoter-linked firefly luciferase] and phRL-TK (thymidin kinase with Renilla luciferase). The last one is the internal control. Twenty-four hours later, cells were washed and treated with live or UV-irradiated RSV [multiplicity of infection (MOI) = 0.5, otherwise indicated], LPS or medium. In some experiments, antibodies against RSV proteins (20 μg ml^{-1}) were added to the cells together with UV-irradiated RSV (MOI = 1.0). The cells were lysed with lysis buffer at the indicated time points and the assay was performed using dual-luciferase reporter assay system. Fold induction against the control medium is indicated at each time point.

Inhibitory effect of the sG protein on the ISRE promoter was tested as follows. The supernatant containing the secreted G protein, UV-irradiated RSV, UV-irradiated MV or medium were added to HEK293 cells, and then cells were transfected with TICAM-1/pEFBos (50 ng), IKKε/pcDNA3.1 (200 ng), TBK1/pcDNA3.1 (200 ng), MAVS/pEFBos (400 ng), ΔRIG-I/pEFBos (700 ng) or MDA5N/pEFBos or pEFBos (700 ng) and 100 ng of pISRE and 3 ng of pRL-TK. Six hours later, cells were harvested with trypsin, washed with PBS and treated with 20 μl of Passive Lysis Buffer. Luciferase activities were measured by Dual-Luciferase assay kit (Promega). The luciferase activity of firefly was normalized by that of Renilla and relative fold activation to the medium control was determined. All experiments were performed in triplicate.

Results

Immune responses induced in human DCs by RSV stimulation

DCs in the respiratory tract play important roles in the immune response against RSV infection. Human mDCs prepared from three healthy donors were incubated with RSV at MOI = 0.5. Viral proteins were detected on the mDC surface within 24 h and kept expressed over 48 h using anti-RSV polyclonal antibodies by FACS analysis (Fig. 1A). Thus, human mDCs are susceptible to RSV of this subgroup B isolate.

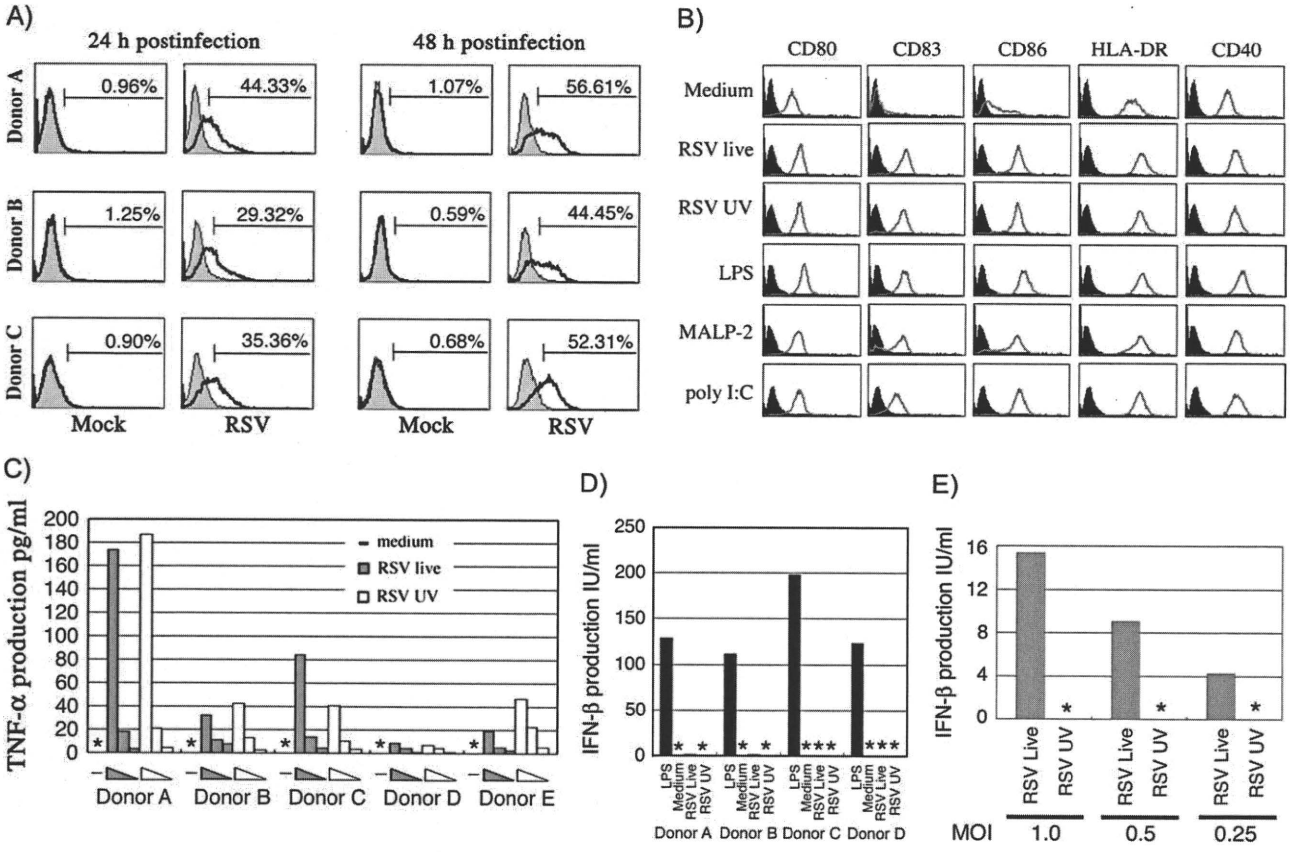

Fig. 1. Human DCs responding to RSV. (Panel A) Human immature mDCs are susceptible to RSV infection. Immature mDCs were incubated with Mock or RSV (MOI = 0.5). These mDCs were stained with FITC-labeled goat anti-RSV polyclonal antibodies or FITC-labeled control mouse Ig 24 or 48 h after RSV infection. %RSV-positive cells are indicated in the FACS histograms. (Panel B) mDC maturation is induced by RSV treatment. Immature mDCs were treated with indicated TLR ligands, medium only or RSV (live or UV irradiated, MOI = 0.5). Twenty-four hours later, mDCs were allowed to react with the indicated antibodies of $F(ab')_2$ against mDC maturation markers (open histograms). Isotype-matched IgG was used as controls (closed histograms). The experiments were performed three times and represented results are shown. (Panel C) Production of TNF-α by mDCs treated with live or UV-irradiated RSV. Human immature mDCs were prepared from five healthy donors and individually treated with RSV (live or UV irradiated) at MOI = 0.5, 0.25 and 0.1. The culture supernatants of the mDCs were harvested in 24 h and the levels of TNF-α determined by ELISA. Asterisk means 'not detected'. (Panels D and E) Production of IFN-β by mDCs in response to RSV. Immature mDCs were treated with RSV (live or UV irradiated) at MOI = 0.5 (otherwise indicated). LPS (100 ng ml^{-1}) or medium were used as controls. Twenty-four hours later, the supernatants were collected, and the levels of IFN-β were measured by ELISA. Asterisk, not detected.

To examine DC maturation by RSV, mDCs were stimulated with live or UV-irradiated RSV, LPS (TLR4 ligand), MALP-2 (TLR2 ligand) or polyI:C (TLR3 ligand) (Fig. 1B). Stimulation with either live or UV-irradiated RSV led to maturation of mDC as determined by cell-surface markers (CD80, CD83, CD86, HLA-DR and CD40) as was the case with the other TLR ligands. Since UV-irradiated RSV induced mDC maturation, RNA replication after viral entry is not a main cause for the RSV-mediated mDC maturation.

Next, we examined if mDCs produce TNF-α and IFN-β in response to RSV infection/stimulation. TNF-α is induced mainly through NF-κB activation and known to mature mDCs. mDCs from various individuals were incubated with the indicated doses of live or UV-irradiated RSV (Fig. 1C). LPS was used as a positive control for the TLR4 ligand. Twenty-four hours later, the supernatants were collected for ELISA. The levels of TNF-α were increased in the supernatant of mDCs in a RSV dose-dependent manner irrespective of RSV treatment, live or UV irradiation (Fig. 1C). Thus, viral attachment to cells rather than replication triggers TNF-α production. However, IFN-β was barely produced in mDCs treated with RSV (Fig. 1D). Although higher doses of live RSV minimally induce IFN-β (<20 IU ml^{-1}) in mDCs during 24 h (i.e. replication dependently, presumably via the cytoplasmic pathway), UV-irradiated RSV did not induce IFN-β by MOI = 1.0 (Fig. 1E) and even at MOI = 5 (data not shown). Although IFN-β induction appears to occur by stimulation of TLR4 with the F protein (30), this is not the case in challenge with UV-irradiated RSV. mDC maturation with TNF-α production but poor IFN-β production is a characteristic phenotype in RSV-affected human mDCs.

Quantitative PCR analysis was performed with mDCs for surveying cytokine induction. UV-inactivated RSV induced a minute amount of the IFN-β message in mDCs but failed to induce it >6 h after stimulation, although live RSV allowed mDCs to induce incremental IFN-β >12 h post-infection (p.i.) (Fig. 2A). In contrast, the TNF-α was somehow kept to be constant >12 h in inactive RSV-stimulated mDCs (Fig. 2B). We consistently found that IFN-inducible genes were barely up-regulated by function of UV-irradiated RSV even after 6 h (data not shown) and 24 h post-stimulation (Fig. 2C). IFN-inducible genes were up-regulated only when mDCs were challenged with high doses of live RSV after 12 h. According to the ~4 h mRNA levels and 24 h ELISA results, RSV-mediated robust IFN induction is the replication-dependent event.

RSV inhibits virus–cell contact-mediated IFN-β induction

UV-inactivated RSV induced TNF-α but barely induced IFN-β in the early phase of mDCs. We asked what causes the impotent production of IFN-β in response to the external stimulation of RSV. We tested the reporter-activating abilities of RSV using the ELAM (for NF-κB) and ISRE (for IFN-β) reporter assays in HEK293 cells. Neither of the promoters was activated in response to UV-irradiated RSV at the indicated time points (Fig. 3A and B). Live RSV on the other hand prominently activated ISRE by ~24 h p.i. (Fig. 3B) and ELAM >12 h p.i. (Fig. 3A). This activation was not due to contaminating LPS since the HEK293 cells did not express TLR4 (Fig. 3A and B). The RSV replication-dependent events will markedly happen >12 h p.i..

A previous report (29) demonstrated that the RSV F protein serves as a TLR4 agonist. Thus, virus–cell contact by live and UV-irradiated RSV should extrinsically activate NF-κB via the TLR4 pathway independent of viral replication. This issue was confirmed with HEK293 cells expressing TLR4 and the stimulation period by 6 h (Fig. 3C and D). ELAM promoter activation was observed in response to live and UV-inactive RSV to a similar extent (Fig. 3C). However, virtually no ISRE activation was detected under this setting (Fig. 3D). Hence, RSV activates the IFN-β promoter in an only replication-competent fashion >24 h p.i.. There is a discrepancy between NF-κB and IFN-β promoter activation.

When HEK293 cells expressing TLR4 were stimulated with LPS and various doses of live or UV-irradiated RSV, RSV dose dependently inhibited LPS-mediated activation of the ISRE promoter (Fig. 4A) irrespective of irradiation, since the analysis was performed within 12 h i.e. before significant viral replication. To test if the inhibition was RSV (but not TLR4) specific, TLR3-expressing HEK293 cells were stimulated with polyI:C in concert with various doses of live or UV-irradiated RSV. Both live and UV-irradiated RSV dose dependently inhibited ISRE promoter activation by polyI:C in terms of TLR3 signaling (Fig. 4B). We confirmed the suppression of IFN-β induction by RSV in mDCs. IFN-β protein production by LPS or polyI:C (determined 24 h p.i.) was also dose dependently inhibited by UV-irradiated RSV in mDCs (Fig. 4C). Function-neutralizing studies were performed using polyclonal antibodies against RSV envelope proteins. We set the conditions where polyI:C induced activation of the ISRE promoter in HEK cells and this activation was partly inhibited in response to live or UV-irradiated RSV that was administered for virus–cell contact (Fig. 4D). A typical result is shown in Fig. 4(D), where the pAb against RSV abrogated RSV-dependent inhibition of ISRE promoter activation. This implies that the virus–cell contact due to a RSV-exposing factor inhibits IFN-β promoter activation in host cells. Since the RSV F protein does not activate TLR3, we used the TLR3/polyI:C system in the following inhibition experiments.

RSV G protein is surface expressed to inhibit the IFN-β pathway

The question is what factor of the RSV envelope proteins participates in inhibition of polyI:C-derived IFN-β induction. Plasmid constructs were generated with the indicated RSV envelope proteins tagged with Flag (Fig. 5A). We confirmed protein expression in HEK293 cells using SDS–PAGE and western blotting with an anti-Flag antibody (Fig. 5B). Under reducing conditions, the F, G and sG proteins were detected on the blot at their expected molecular masses (Fig. 5B). Under non-reducing conditions, all these proteins tended to form multimers. In particular, the SH and F proteins formed multimers, which were partially dissociated upon heating and reduction (Fig. 5B), consistent with a previous report (28). The F, SH and G proteins, but not sG proteins, were N-glycosylated and no high mannose was detected on these

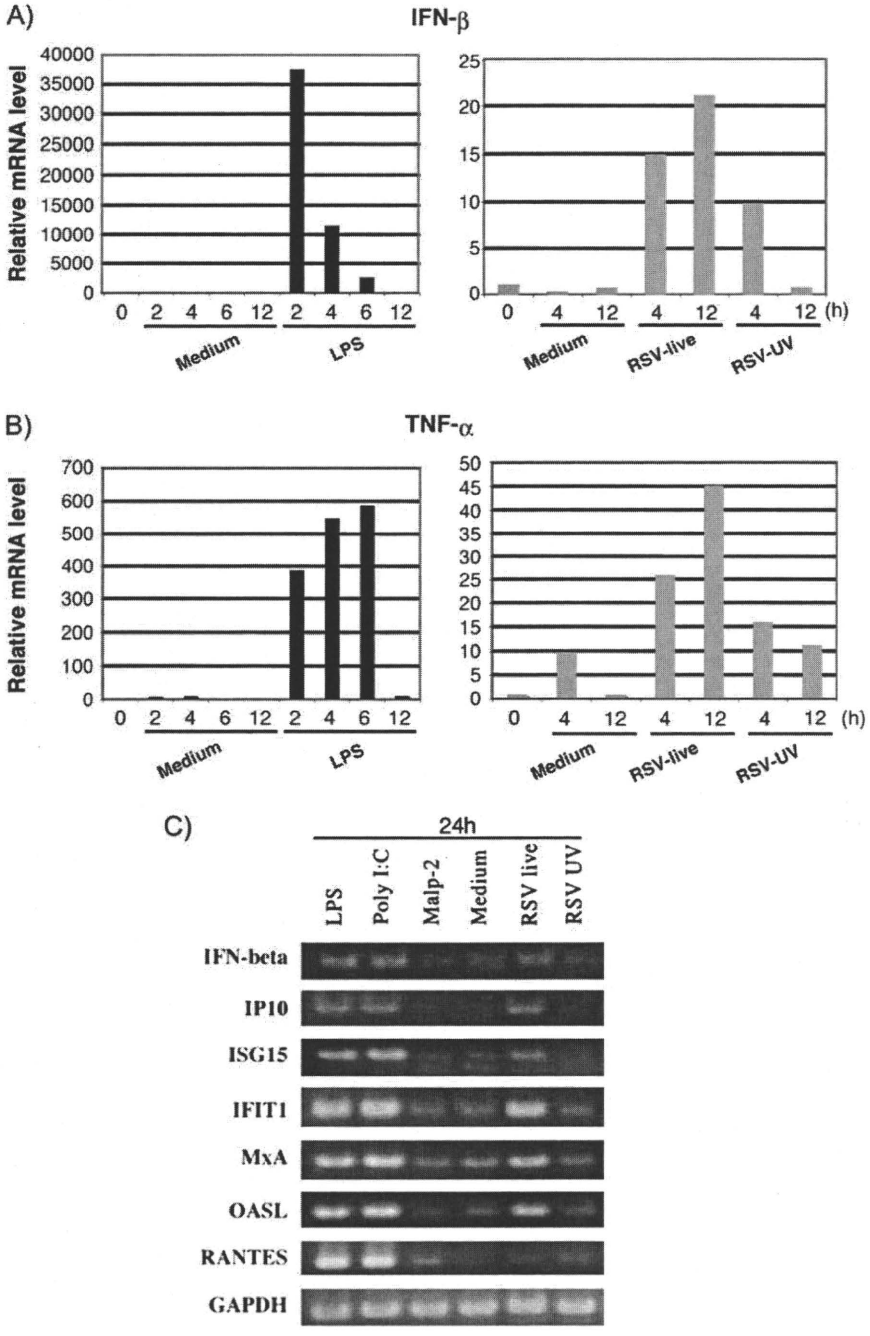

Fig. 2. Early induction of minute amounts of IFN-βthrough RSV–mDC interaction. (Panels A and B) Early induction of TNF-αand IFN-βby mDCs in response to RSV attachment. Human immature mDCs were treated with LPS (positive control), medium only (negative control) or live or UV-irradiated RSV (MOI = 0.5) as in Fig. 1(E). At indicated timed intervals, mRNA was harvested from the treated mDCs. Quantitative PCR was performed with these RNA samples pertaining to the cytokines indicated. (Panel C) Live RSV infects human mDCs and causes TLR3-independent induction of IFN-inducible genes. Human mDCs were stimulated with the reagents (indicated a top of the panel). The same lot of RSV (MOI = 0.5) as in Fig. 1(E)was used. Twenty-four hours later, mRNA levels of the indicated genes were assessed by reverse transcription–PCR. GAPDH is a control.

proteins (data not shown). Susceptibility of these proteins to glycosidases suggested that these proteins are expressed naturally on HEK293 cells. In addition, a soluble form of the G protein of 48 kDa with no high mannose or N-linked sugars was detected in the supernatant of the cells (Fig. 5C), consistent with a previous notion (33).

Cells were transfected with the indicated expression plasmid, together with TLR3-expressing plasmid and the reporter plasmids. Then, the cells were stimulated with polyI:C and after 6 h, the ISRE reporter activity was measured. G protein derivatives showed a weak ability (usually ~20%) to suppress the polyI:C-mediated ISRE reporter activity as

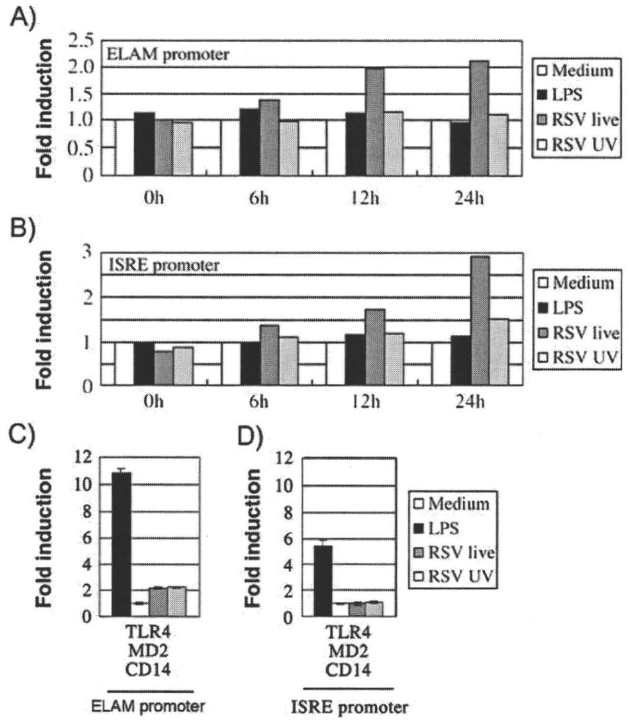

Fig. 3. Replication-dependent promoter activation by live RSV. (Panels A and B) Live but not UV-irradiated RSV activates the ELAM and ISRE promoter. HEK293 cells were transfected with pGV-E2/huELAM or pISRE-Luc and treated with the indicated stimulants as in Fig. 1(E). At timed intervals, luciferase reporter activity was determined for ELAM (A) and ISRE (B) activation. (Panels C and D) HEK293 cells with the ELAM or ISRE reporters were transfected with the plasmid set for TLR4 expression. Twenty-four hours later, cells were washed and treated with RSV (MOI = 0.5), LPS or medium. Six hours after incubation, reporter assay was performed as for ELAM (C) and ISRE (D). In either case, fold induction against medium is indicated. One of three similar experiments is shown.

compared with the vector transfectant (Fig. 6A). With the exception of the G protein, polyI:C-dependent ISRE activation was not affected by the expression of the RSV envelope proteins (Fig. 6A). The result was reproducible under the conditions where proteins were expressed to similar levels. Over-expressing RSV sG protein appears to externally inhibit the TLR3-mediated IFN-β-inducing event.

Since the sG protein maintained its inhibitory effect, we examined the ISRE inhibition by increasing doses of RSV sG protein using the TLR3 or TLR4 system. The culture supernatants from HEK293 cells transfected with the RSV sG (sGncFlag) plasmid were collected as a source of sG and used in the reporter assay. The supernatant of HEK293 cells with vector only was similarly prepared as a control. Cells were transfected with relevant plasmids for the TLR assay, and after 24 h, the cells were stimulated with LPS, polyI:C or media (presence or absence of the RSV sG protein for 6 h (Fig. 6B and C). ISRE activation by LPS–TLR4 (Fig. 6B) and polyI:C–TLR3 (Fig. 6C) were clearly inhibited by the exogenously added sG protein in a dose-dependent manner. These data suggest that it is the G protein that inhibits the TLR3/4-mediated IFN-β-inducing pathway.

Exogenously added RSV G protein suppresses IFN-β production in mDCs

It remained undetermined whether the extrinsic G protein physiologically controls mDC function. We determined IFN-β production in mDCs by stimulation with sG protein. The sG-mediated suppression of IFN production was endorsed with mDCs stimulated with polyI:C or LPS using ELISA (Fig. 7A). Finally, the purified F-protein-mediated IFN-β production was also blocked by RSV-G protein (Fig. 7B). Using the early-phase IFN-β mRNA determination assay by quantitative PCR (Fig. 2A), we checked whether exogenously added sG protein has an ability to inhibit RSV-mediated early (<2 h) induction of the IFN-β message in mDCs. The conditioned medium containing sG protein, if pre-incubated with mDCs, partially suppressed the increase of the IFN-β message by live and UV-irradiated RSV up to 4 h p.i. in mDCs (data not shown). Hence, additional sG protein can modulate mDC functions including IFN-β induction raised secondary to RSV–mDC interaction.

The sG protein selectively inhibits TICAM-1/TRIF signaling

Recent reports described the presence of TLR-independent dsRNA-mediated type I IFN-inducing pathways (38). RIG-I and MDA5 are the sensors responsible for virus RNA recognition (39). These molecules reside in the cytoplasm where they recognize dsRNA or viral RNA-specific patterns and activate IKKε and TBK1 through the adaptor MAVS (IPS-1) (18). To examine whether the sG protein could inhibit the cytoplasmic pathway, we transfected HEK293 cells with reporter plasmids together with the plasmids for the constitutively active form of RIG-I or MDA5 (ΔRIG-I or MDA5N), IPS-1, TICAM-1, IKKε or TBK-1. The IFN-inhibitory effect of the sG protein, UV-irradiated RSV and UV-irradiated MV (as a control) was assessed using the reporter assay after 6 h. The sG protein and UV-irradiated RSV inhibited ISRE activity by TICAM-1, but not by other cytoplasmic factors including RIG-I and MAVS (Fig. 8A). The TICAM-1-mediated ISRE activation was inhibited in a sG dose-dependent manner (Fig. 8B). UV-irradiated MV did not inhibit ISRE activation in terms of all the transfected constructs (Fig. 8A). IKKε/TBK-1-mediated ISRE response was not affected by the sG protein added and rather increased by stimulation with UV-irradiated RSV (data not shown), although the latter reason as yet unknown. Hence, we can conclude that the sG protein selectively inhibits the TICAM-1-signaling pathway upstream of the IRF-3 kinases, but not the RIG-I/MDA5 pathway. This issue was confirmed using TICAM-1- and MAVS-silencing HeLa cells made by the RNAi technology (M. Matsumoto and T. Seya, unpublished data).

Discussion

We demonstrated that mDCs produce only minute amounts of IFN-β in response to live and UV-irradiated RSV while mDCs induce TNF-α to mature in response to the same RSV treatment. IFN-β is poorly produced only when whole-virus particles exogenously attack for mDC infection. This situation may coincide with RSV-mediated mDC maturation which is also triggered by RSV attachment to the host cell surface.

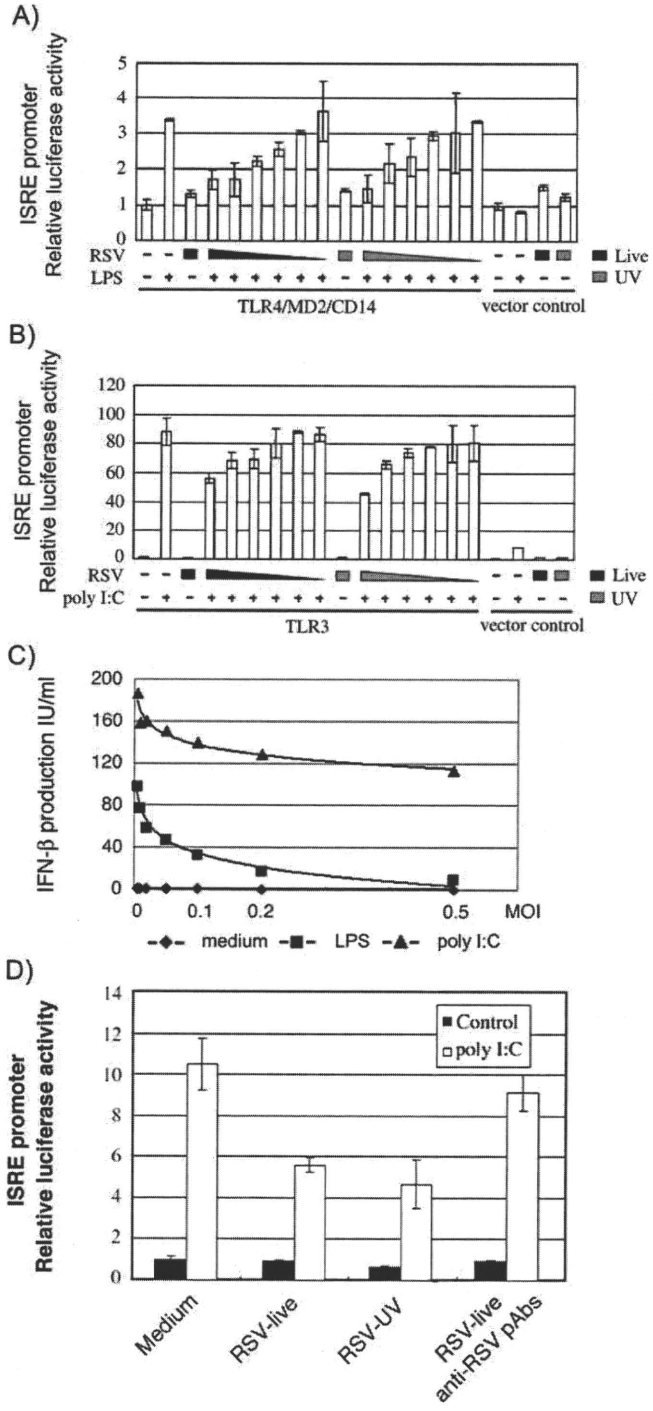

Fig. 4. RSV inhibits TLR-dependent IFN-β induction. (Panels A and B) RSV inhibits reporter activation by ISRE promoter. HEK293 cells were transfected with pISRE-Luc and phRL-TK plasmid, together with the pEFBos TLR4 plasmid sets (TLR4, MD-2 and CD14) (13) or TLR3 plasmid. Twenty-four hours later, cells were washed and treated with various doses of RSV (MOI = 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01) in the presence or absence of LPS (A) or polyI:C (B). Six hours after incubation, cell lysates were subjected to the assay for the dual reporters (13). Fold induction against the medium control is indicated. (Panel C) Immature mDCs were treated with UV-irradiated RSV (MOI indicated) together with LPS or polyI:C. Twenty-four hours later, culture supernatants were collected and the levels of IFN-β measured by ELISA. (Panel D) RSV-mediated inhibition of ISRE promoter activation is abrogated by the addition of pAbs against RSV. HEK293 cells with TLR3 and ISRE promoter were treated with polyI:C (medium) and live (RSV-live) or inactive RSV (RSV-UV) (as in B). Under the conditions where the RSV-mediated inhibition was observed, pAbs against RSV were added to the cells (RSV-live, anti-RSV pAbs). Six hours after incubation, cell lysates were subjected to the assay for the dual reporters.

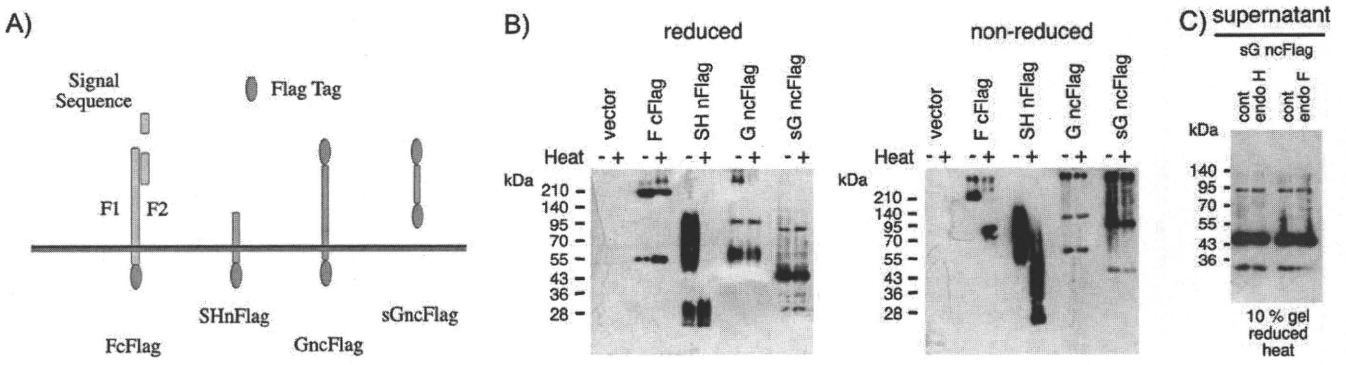

Fig. 5. Detection of the RSV envelope proteins expressed on HEK293 cells. (Panel A) Scheme of the Flag-tagged RSV proteins. Predicted proteins are shown based on the constructs we prepared. F, SH, G and sG are RSV envelope proteins. Flag-labeled (N-terminal, n and/or C-terminal, c) is indicated. Elliptic circles indicate the Flag-tag. FcFlag, C-terminal-flagged F protein; SHnFlag, N-terminal-flagged SH protein; GncFlag, both N- and C-terminal-flagged G protein; sGncFlag, N- and C-terminal-flagged secreted G protein. (Panel B) Immunoblotting analysis of RSV envelope proteins in HEK293 cells. Cells were transfected with the plasmids encoding the RSV envelope proteins tagged with Flag (see A). Twenty-four hours later, cell lysates were immunoprecipitated with anti-Flag antibody and the proteins were resolved on SDS–PAGE (10% gel) under reducing (left panel) or non-reducing conditions (right panel). After protein blotting onto a sheet, blots were probed with anti-Flag pAb. (Panel C) Glycosylation of RSV proteins liberated from HEK cells. The supernatants of the sGncFlag-transfected HEK293 cells in (B) were treated with endoglycosidase H (endo H) or endoglycosidase F (endo F) and analyzed on SDS–PAGE followed by immunoblotting. The conditions of the analyzed samples are shown in the panel.

We found RSV inducing minimal IFN-β through virion–cell attachment (usually taking <4 h p.i.) and then inducing robust IFN-β after cytoplasmic replication (>12 h p.i.). The F protein should be an effector for the RSV-mediated IFN-β induction, but somehow the IFN-β induction tends to be diminished in RSV–host cell interaction. We searched for the factor negatively regulating IFN-β induction in host HEK293 cells using UV-inactive RSV and found that attachment of RSV envelope proteins to host cells causes down-regulation of IFN-β. Finally, the G protein of RSV is the factor for the inhibition of IFN-β promoter activation: even by the stimulation with polyI:C or LPS, bystander inhibition happens by function of the soluble form of the G protein (sG). Addition of the sG protein to the culture of mDCs allows the suppression of polyI:C- or LPS-mediated IFN-β production. Ultimately, here we disclose a novel function of the RSV G protein in the regulation of host cell IFN response.

Using *in vitro* analysis, we found that the RSV F protein-mediated IFN induction (30) is neutralized by the RSV G protein, which selectively modulates the TICAM-1 pathway, i.e. the preferential activation of IRF-3 and the IFN-β promoter in myeloid DCs. The G protein can inhibit both TLR3 and TLR4 to suppress IFN-β induction, supporting the target TICAM-1. Studies using reporter analysis, ELISA with mDCs and gene-silencing analysis of MAVS and TICAM-1 using RNAi (M. Matsumoto and T. Seya, unpublished data) all supported the G protein function in the TICAM-1 pathway.

How the G protein modulates the TICAM-1-mediated IFN-inducing function remains as a tantalizing point in this story. A possible molecular mechanism is that the G protein is produced after replication and a putative receptor for the G protein delivers a negative signal to the TICAM-1 pathway. This G protein receptor may exist in the cytoplasmic compartment or on cell surface and link the TICAM-1 pathway in the cytoplasm. This hypothesis may be related to the fact that a defective recombinant virus lacking the sG protein decreases the virus pathogenicity due to the induction of antiviral immunity (40). In addition, how dsRNA stimulation activates TICAM-1 is getting clear in a molecular level (41). Furthermore, a recent report (42) suggested that the TIR-containing adaptor SARM exhibits a regulatory function toward TICAM-1. Further studies are required to clarify the mode for TICAM-1 inhibition by extrinsic G proteins.

Since IFN-inducible genes are significantly up-regulated in mDCs in response to live RSV after 24 h p.i. (Fig. 2C), the initial trigger of IFN induction by live RSV may be too weak to suppress RSV replication, so that the infected mDCs elicit following replication-mediated response. In fact, importance of 'revving up' activation of IFN-β for amplifiable IFN-α/β response has been proposed in a recent review (43). RIG-I and MDA5 are preferentially responsible for the replication-dependent antiviral event in response to live viruses, which is evident in the airway epithelial cells (6). Since RIG-I and MDA5 are IFN-inducible proteins, an initial trigger of IFN-β also critically causes their induction in virus-attached cells. We surmise the importance of F protein-mediated TLR4 signal in an early response of cells to RSV. Blocking of the F protein function by G proteins may be crucial for silencing the IFN-inducing response and for the virus side facilitating RSV infection. Indeed, immature mDCs secrete TNF-α and mature in a similar manner in response to both live and dead RSV, possibly reflecting minute participation of type I IFN in the RSV-mediated maturation phenotype of mDCs.

The difference in outcome between TLR and RIG-I/MDA5 signaling is an intriguing question. TLR3 senses viral RNA outside the cytoplasm and RIG-I/MDA5 sense it inside the cytoplasm. RIG-I/MDA5 and TLR3 recruit different adaptors, IPS-1 and TICAM-1, respectively. Although TICAM-1 and IPS-1 interact partly with TANK family proteins (10, 44), only the TICAM-1 pathway is reported to elicit potencies to activate CTL and NK cells in mDCs (11, 12). Our premise is that viral RNA replication inside the cytoplasm and extrinsic dsRNA stimulation lead to differential mDC driving. Selective inhibition

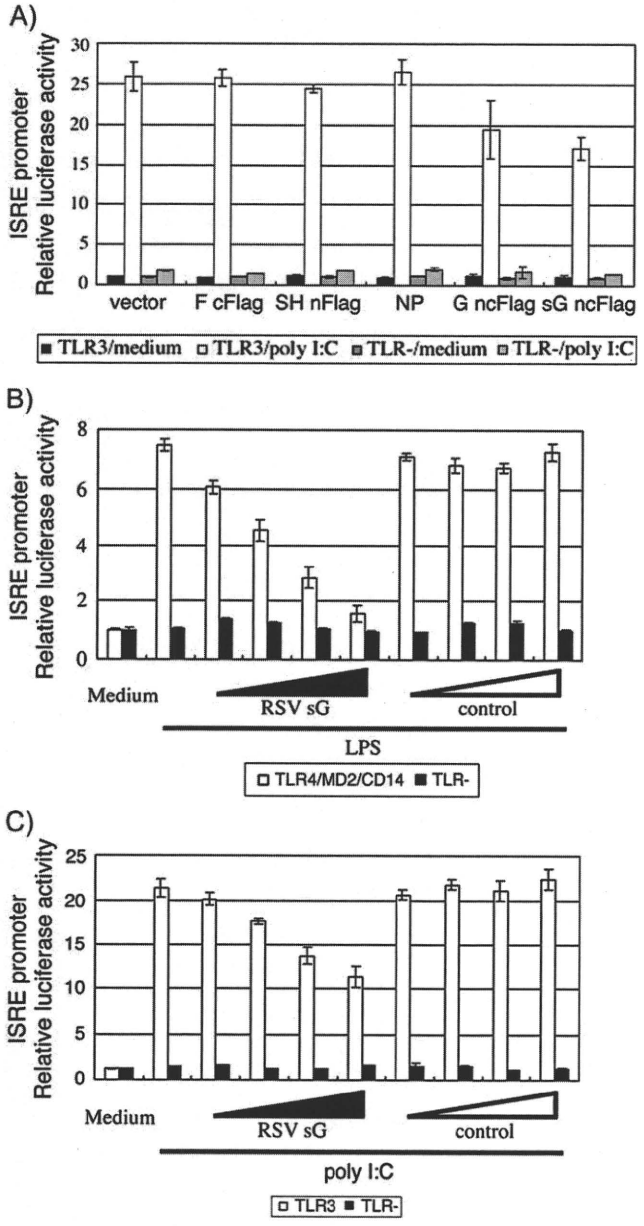

Fig. 6. RSV G protein inhibits activation of the ISRE promoter. (Panel A) RSV G protein inhibits ISRE activation by TLR3. HEK293 cells were transfected simultaneously with pISRE-Luc, phRL-TK, pEFBos TLR3 and indicated plasmids encoding RSV proteins. Twenty-four hours later, cells were incubated with 10 μg ml^{-1} of polyI:C or buffer only. After 6 h, dual-luciferase reporters were assayed as in Fig. 4(A). (Panel B and C) sG protein inhibits ISRE activation by LPS or polyI:C. HEK293 cells expressing TLR4/MD-2/CD14 or TLR3 were prepared and then the ligand stimulation was added to the cells in the medium containing RSV sG. HEK293 cells were transfected with pISRE-Luc, phRL-TK and pEFBos TLR4 expression plasmids or TLR3 plasmid. Twenty-four hours later, cells were stimulated with 100 ng ml^{-1} of LPS (B) or 10 μg ml^{-1} of polyI:C (C) under various doses of RSV sG (1/5, 1/10, 1/20 and 1/40 volumes of medium). The culture supernatant from the empty vector-transfected cells was used as a control. After 6 h incubation, luciferase reporter activity was measured as in Fig. 4(A). The figures are representative results of multiple trials.

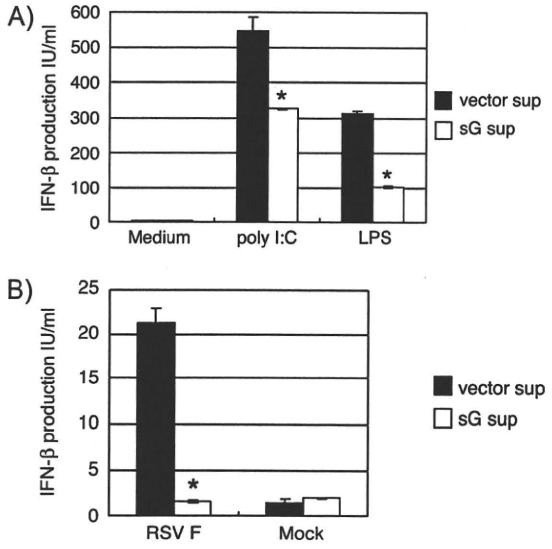

Fig. 7. RSV sG protein inhibits IFN-β production by mDCs stimulated with polyI:C or RSV F protein. (Panel A) sG protein inhibits polyI:C-inducing IFN-β production in human mDCs. mDCs were prepared as in Fig. 1. Cells were stimulated with polyI:C in the presence of the sG protein-containing or control medium. Twenty-four hours later, the supernatants were harvested to measure the IFN-β content. LPS was used as control as in Fig. 2(D). (Panel B) Purified F protein allows mDCs to produce IFN-β, which is inhibited by sG. Immature mDCs were stimulated with the purified F protein (1 μg) in the presence or absence of the sG-containing medium. Twenty hours later, the IFN-β levels released in the supernatant of mDCs were determined by ELISA.

of the TICAM-1 pathway may benefit RSV survival and happen to suppress mDC-derived cellular immune responses. Severe repetitive infection by RSV occurring in children and being referred to insufficient mDC maturation, may be partly due to this extrinsic mDC regulation by RSV proteins.

The question is whether the early IFN induction via RSV-attached host cells is physiologically significant in mDCs. A number of RSV studies have suggested that TLR3 is implicated in the immune response of epithelial cells. IL-8, RANTES, TNF-α and IL-6 are up-regulated secondary to RSV infection (45–47). In addition, IFN-inducible genes, including *TLR3* and *PKR*, are up-regulated (5). These findings were reported before the molecular identity of RIG-I/MDA5 was completed and were based on the assumption that the source of dsRNA was from RSV RNA released from cells undergoing infection-induced apoptosis. It is still unclear whether the virus–cell attachment-mediated TICAM-1 blocking earlier and more significantly participates in initial IFN induction than the intrinsic cytoplasmic IFN-inducing pathway. However, in RSV infection, this G protein-mediated TICAM-1 blocking would be crucial since RSV possesses the TLR4 ligand F protein. The question is whether these findings are adaptable to human patients with RSV infection. Further analysis will be required about what happens in mDCs and acquired immunity once replication-derived viral RNA products are generated in patients' body (8, 9).

Regarding viral aspects, a recent report suggested that the G cysteine-rich region of the RSV sG protein inhibits production of NF-κB-inducible inflammatory cytokines through TLR4 (48). Since MyD88 is not a target of the RSV G

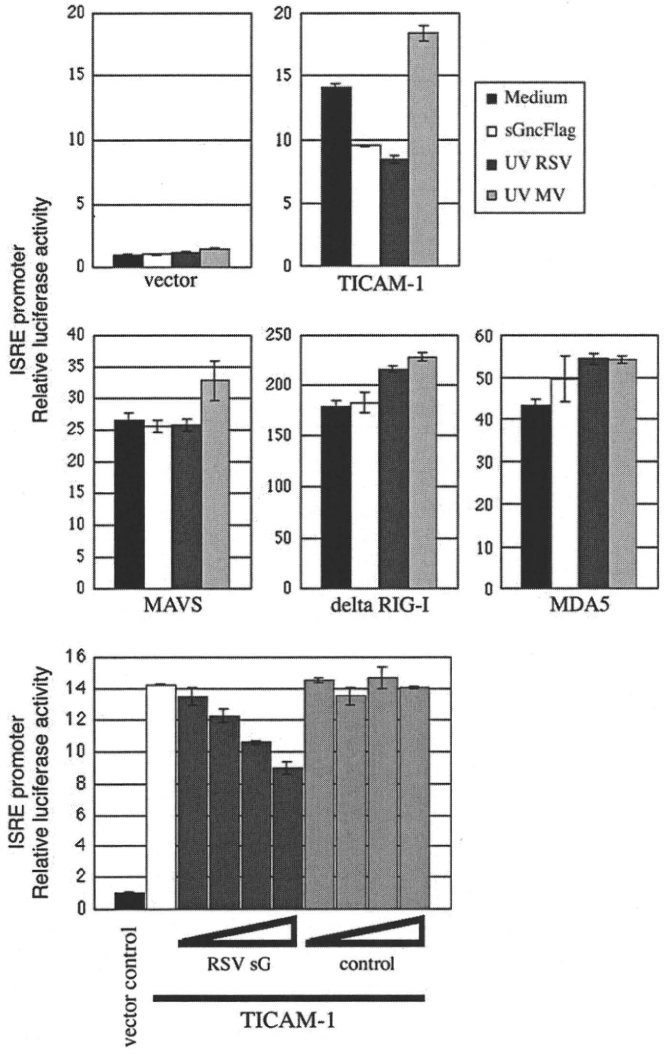

Fig. 8. The sG protein inhibits the TICAM-1 pathway. (Panel A) The sG protein blocks TICAM-1-mediated ISRE promoter activation. HEK293 cells were treated with a control medium or medium containing sG along with control dead RSV or MV and transfected with pISRE-Luc, phRL-TK and the plasmids expressing for the indicated proteins. Six hours later, cells were washed, lysed with lysis buffer and the reporter assay was performed as in Fig. 4(A). (Panel B) The sG protein dose dependently inhibits the TICAM-1 pathway. HEK293 cells were transfected with the TICAM-1 plasmid, and the TICAM-1-mediated ISRE promoter activation was monitored in the presence of variable amounts of the RSV sG-containing medium (1/5, 1/10, 1/20 and 1/40 volumes of medium). The culture supernatant from the empty vector-transfected cells was used as a control.

proteins in NF-κB activation (data not shown), the G protein can distinguish between MyD88 and TICAM-1 as the molecular target. Besides the RSV F protein, many viral envelope proteins are known to act as ligands for TLR2 or TLR4. In general, many viral proteins reportedly inhibit the JAK/STAT pathway and IRF-3 activation. NS3/4A of HCV inactivates IPS-1 and TICAM-1 by proteolysis (49). Vaccinia virus proteins also target TLR adaptor proteins (50). RSV NS1 and NS2 are simultaneously generated with viral RNA in the cytoplasm. These proteins act as inhibitors for IFN-α/β signaling after replication (26, 27). Here, we add to these findings a line of evidence that the RSV G protein is a negative regulator for the TLR3/4-mediated TICAM-1 pathway.

Funding

CREST and Innovation; Japan Science and Technology Corporation; Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases; MEXT; Ministry of Education, Science, and Culture (Specified Project for Advanced Research); Ministry of Health, Labor and Welfare of Japan.

Acknowledgements

We are grateful to A. Ishii and A. Matsuo in our laboratory for their critical discussions. Thanks are also due to K. Imai (Wakayama Prefectural Center, Wakayama) for providing us with RSV, to T. Taniguchi (University of Tokyo, Tokyo), T. Fujita (Kyoto University, Kyoto), K. Miyake (University of Tokyo, Tokyo), M. Nakanishi (The Nagoya City University, Nagoya) and T. Maniatis (Harvard University, Boston, MA, USA) for providing their plasmids. Support by Takeda Science Foundation and NorthTec Foundation are gratefully acknowledged.

Abbreviations

DC	dendritic cell
dsRNA	double-stranded RNA
F protein	fusion glycoprotein
G protein	G glycoprotein
IKKε	IκB kinase-related kinase ε
IPS-1	IFN-β promoter stimulator 1
IRF	IFN-regulatory factor
MALP-2	macrophage-activating lipopeptide-2
MAVS	mitochondria antiviral signaling
MDA5	melanoma differentiation-associated gene 5
mDC	monocyte-derived dendritic cell
MOI	multiplicity of infection
MV	measles virus
NF-κB	nuclear factor-κB
p.i.	post-infection
RIG-I	retinoic acid-inducible gene I
RSV	respiratory syncytial virus
sG	soluble G
TBK1	TANK-binding kinase 1
TIR	Toll–IL-1R
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α

References

1 Ogra, P. L. 2004. Respiratory syncytial virus: the virus, the disease and the immune response. *Paediatr. Respir. Rev.* 5:119.
2 Steinman, R. M. and Hemmi, H. 2006. Dendritic cells: translating innate to adaptive immunity. *Curr. Top. Microbiol. Immunol.* 311:17.
3 Srikiatkhachorn, A. and Braciale, T. J. 1997. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. *J. Exp. Med.* 186:421.
4 Creagh, E. M. and O'Neill, L. A. 2006. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol.* 27:352.
5 Groskreutz, D. J., Monick, M. M., Powers, L. S., Yarovinsky, T. O., Look, D. C. and Hunninghake, G. W. 2006. Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. *J. Immunol.* 176:1733.
6 Liu, P., Jamaluddin, M., Li, K., Garofalo, R., Casola, A. and Brasier, A. 2007. Retinoic acid inducible gene-I mediates early anti-viral

response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J. Virol.* 81:1401.
7 Rudd, B. D., Burstein, E., Duckett, C. S., Li, X. and Lukacs, N. W. 2005. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. *J. Virol.* 79:3350.
8 Hornung, V., Ellegast, J., Kim, S. *et al.* 2006. 5′-Triphosphate RNA is the ligand for RIG-I. *Science* 314:994.
9 Pichlmair, A., Schulz, O., Tan, C. P. *et al.* 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* 314:997.
10 Sasai, M., Shingai, M., Funami, K. *et al.* 2006. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in Type I IFN induction. *J. Immunol.* 177:8676.
11 Akazawa, T., Ebihara, T., Okuno, M. *et al.* 2007. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proc. Natl Acad. Sci. USA* 104:252.
12 Schulz, O., Diebold, S. S., Chen, M. *et al.* 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433:887.
13 Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. and Seya, T. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 4:161.
14 Yamamoto, M., Sato, S., Mori, K. *et al.* 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J. Immunol.* 169:6668.
15 Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M. and Seya, T. 2003. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. *J. Biol. Chem.* 278:49751.
16 Yamamoto, M., Sato, S., Hemmi, H. *et al.* 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* 4:1144.
17 Matsumoto, M. and Seya, T. 2008. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv. Drug Deliv. Rev.* 60:805.
18 Kawai, T., Takahashi, K., Sato, S. *et al.* 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 6:981.
19 Meylan, E., Curran, J., Hofmann, K. *et al.* 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437:1167.
20 Seth, R. B., Sun, L., Ea, C. K. and Chen, Z. J. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122:669.
21 Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z. and Shu, H. B. 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol. Cell* 19:727.
22 Takeuchi, O. and Akira, S. 2008. MDA5/RIG-I and virus recognition. *Curr. Opin. Immunol.* 20:17.
23 Honda, K., Yanai, H., Negishi, H. *et al.* 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434:772.
24 Uematsu, S., Sato, S., Yamamoto, M. *et al.* 2005. Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. *J. Exp. Med.* 201:915.
25 Hoshino, K., Sugiyama, T., Matsumoto, M. *et al.* 2006. IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. *Nature* 440:949.
26 Gotoh, B., Komatsu, T., Takeuchi, K. and Yokoo, J. 2001. Paramyxovirus accessory proteins as interferon antagonists. *Microbiol. Immunol.* 45:787.
27 Ramaswamy, M., Shi, L., Varga, S. M., Barik, S., Behlke, M. A. and Look, D. C. 2006. Respiratory syncytial virus nonstructural protein 2 specifically inhibits type I interferon signal transduction. *Virology* 344:328.
28 Collins, P. L. and Mottet, G. 1991. Post-translational processing and oligomerization of the fusion glycoprotein of human respiratory syncytial virus. *J. Gen. Virol.* 72:3095.
29 Kurt-Jones, E. A., Popova, L., Kwinn, L. *et al.* 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat. Immunol.* 1:398.
30 Rudd, B. D., Luker, G. D., Luker, K. E., Peebles, R. S. and Lukacs, N. W. 2007. Type I interferon regulates respiratory virus infected dendritic cell maturation and cytokine production. *Viral Immunol.* 20:531.
31 Levine, S., Klaiber-Franco, R. and Paradiso, P. R. 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. *J. Gen. Virol.* 68:2521.
32 Bukreyev, A., Whitehead, S. S., Murphy, B. R. and Collins, P. L. 1997. Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-specific attenuation in the respiratory tract of the mouse. *J. Virol.* 71:8973.
33 Hendricks, D. A., Baradaran, K., McIntosh, K. and Patterson, J. L. 1987. Appearance of a soluble form of the G protein of respiratory syncytial virus in fluids of infected cells. *J. Gen. Virol.* 68:1705.
34 Tripp, R. A. 2004. Pathogenesis of respiratory syncytial virus infection. *Viral Immunol.* 17:165.
35 Nishiguchi, M., Matsumoto, M., Takao, T. *et al.* 2001. 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.* 166:2610.
36 Ebihara, T., Masuda, H., Akazawa, T. *et al.* 2007. NKG2D ligands are induced on human dendritic cells by TLR ligand stimulation and RNA virus infection. *Int. Immunol.* 19:1145.
37 Peret, T. C., Hall, C. B., Schnabel, K. C., Golub, J. A. and Anderson, L. J. 1998. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. *J. Gen. Virol.* 79:2221.
38 Matsumoto, M., Seya, T. and Nagasawa, S. 1992. Polymorphism and proteolytic fragments of granulocyte membrane cofactor protein (MCP, CD46) of complement. *Biochem. J.* 281:493(Pt 2).
39 Yoneyama, M. and Fujita, T. 2007. Function of RIG-I-like receptors in antiviral innate immunity. *J. Biol. Chem.* 282:15315.
40 Maher, C. F., Hussell, T., Blair, E., Ring, C. J. and Openshaw, P. J. 2004. Recombinant respiratory syncytial virus lacking secreted glycoprotein G is attenuated, non-pathogenic but induces protective immunity. *Microbes Infect.* 6:1049.
41 Funami, K., Sasai, M., Oshiumi, H., Seya, T. and Matsumoto, M. 2008. Homo-oligomerization is essential for Toll/Interleukin-1 receptor domain-containing adaptor molecule-1-mediated NF-kB and IRF-3 activation. *J. Biol. chem.* 283:18283.
42 Carty, M., Goodbody, R., Schroder, M., Stack, J., Moynagh, P. N. and Bowie, A. G. 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat. Immunol.* 7:1074.
43 Takaoka, A. and Taniguchi, T. 2003. New aspects of IFN-alpha/beta signalling in immunity, oncogenesis and bone metabolism. *Cancer Sci.* 94:405.
44 Ryzhakov, G. and Randow, F. 2007. SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. *EMBO J.* 26:3180.
45 Olszewska-Pazdrak, B., Casola, A., Saito, T. *et al.* 1998. Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. *J. Virol.* 72:4756.
46 Panuska, J. R., Merolla, R., Rebert, N. A. *et al.* 1995. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. Suppression of early cytokine production and implications for incomplete immunity. *J. Clin. Invest.* 96:2445.
47 Tsutsumi, H., Matsuda, K., Sone, S., Takeuchi, R. and Chiba, S. 1996. Respiratory syncytial virus-induced cytokine production by neonatal macrophages. *Clin. Exp. Immunol.* 106:442.
48 Polack, F. P., Irusta, P. M., Hoffman, S. J. *et al.* 2005. The cysteine-rich region of respiratory syncytial virus attachment protein inhibits innate immunity elicited by the virus and endotoxin. *Proc. Natl Acad. Sci. USA* 102:8996.
49 Li, K., Foy, E., Ferreon, J. C. *et al.* 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl Acad. Sci. USA* 102:2992.
50 O'Neill, L. A. and Bowie, A. G. 2007. The family of five: tIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* 7:353.

Hepatol Int
DOI 10.1007/s12072-008-9055-4

ORIGINAL ARTICLE

Increased expression of Toll-like receptor 3 in intrahepatic biliary epithelial cells at sites of ductular reaction in diseased livers

Minoru Nakamura · Kenji Funami · Atsumasa Komori · Terufumi Yokoyama · Yoshihiro Aiba · Aiko Araki · Yasushi Takii · Masahiro Ito · Mutsumi Matsuyama · Makiko Koyabu · Kiyoshi Migita · Ken Taniguchi · Hikaru Fujioka · Hiroshi Yatsuhashi · Misako Matsumoto · Hiromi Ishibashi · Tsukasa Seya

Received: 23 August 2007 / Accepted: 23 January 2008

Abstract *Background* Toll-like receptors (TLRs) may play active roles in both innate and adaptive immune responses in human intrahepatic biliary epithelial cells (HIBECs). The role of TLR3 expressed by HIBECs, however, remains unclear. *Methods* We determined the in vivo expression of TLRs in biopsy specimens derived from diseased livers immunohistochemically using a panel of monoclonal antibodies against human TLRs. We then examined the response of cultured HIBECs to a TLR3 ligand, polyinosinic–polycytidylic acid (polyI:C). Using siRNAs specific for Toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1) and mitochondrial antiviral signaling protein (MAVS), we studied signaling pathways inducing IFN-β expression. *Results* The expression of TLR3 was markedly increased in biliary epithelial cells at sites of ductular reaction in diseased livers, including primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), and chronic viral hepatitis (CH) as compared to nondiseased livers. Although cultured HIBECs constitutively expressed TLR3 at both the protein and mRNA levels in vitro, the addition of polyI:C to culture media induced only minimal increases in IFN-β mRNA. In contrast, transfection of HIBECs with polyI:C induced a marked increase in mRNAs encoding a variety of chemokines/cytokines, including IFN-β, IL-6, and TNF-α. The induction of IFN-β mRNA was efficiently inhibited by an siRNA against MAVS but not against TICAM-1, indicating that the main signaling pathway for IFN-β induction following polyI:C transfection is via retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5) in HIBECs. *Conclusions* TLR3 expression by biliary epithelial cells increased at sites of ductular reaction in diseased livers; further study will be necessary to characterize it's in vivo physiological role.

Keywords Primary biliary cirrhosis (PBC) · Human intrahepatic biliary epithelial cells (HIBECs) · Interferon beta (IFN-β) · Toll-like receptor 3 (TLR3) Toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1) · Mitochondrial antiviral signaling protein (MAVS) · Retinoic acid inducible gene I (RIG-I) · Melanoma differentiation-associated gene 5 (MDA5)

Abbreviations

BEC	Biliary epithelial cell
CK	Cytokeratin
dsRNA	Double stranded RNA
ER	Endoplasmic reticulum
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HIBEC	Human intrahepatic biliary epithelial cell
HRP	Horseradish peroxidase
IFN	Interferon

M. Nakamura (✉) · A. Komori · T. Yokoyama · Y. Aiba · A. Araki · Y. Takii · M. Ito · M. Matsuyama · M. Koyabu · K. Migita · K. Taniguchi · H. Fujioka · H. Yatsuhashi · H. Ishibashi
Clinical Research Center, National Hospital Organization (NHO) Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan
e-mail: nakamuram@nmc.hosp.go.jp

M. Nakamura
Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan

K. Funami · M. Matsumoto · T. Seya
Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

IL	Interleukin
IRF	Interferon regulatory factor
MAVS	Mitochondrial anti-viral signaling protein
MDA5	Melanoma differentiation associated gene-5
MyD88	Myeloid differentiation factor 88
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cells
PolyI:C	Polyinosinic–polycytidylic acid
PRR	Pattern-recognition receptor
RIG-I	Retinoic acid-inducible gene I
RT-PCR	Reverse transcription-polymerase chain reaction
siRNA	Small interfering RNA
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TICAM-1	Toll-IL-1R homology domain containing adaptor molecule 1

Introduction

Epithelial cells are the first barrier against viral infection. Such cells typically express retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5) and Toll-like receptor 3 (TLR3) to sense double-stranded RNAs (dsRNA), hallmarks of viral replication [1–3]. TLR3 is localized to endosomes and/or the cell surface in epithelial cells, while RIG-I/MDA5 resides in the cytoplasm [3–5]. TLR3-expressing epithelial cells are widely distributed throughout the body, with prominent expression in intestinal, cervical, uterine, endometrial, bronchial, and corneal epithelial cells, the central nervous system, and epidermal keratinocytes [6–16]. The function of TLR3 has been intensively studied in some of these epithelial cells; bronchial epithelial cells recognize dsRNA by cell-surface TLR3 and induce cellular responses, including the secretion of type 1 interferon (IFN) via the Toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1)-interferon regulatory factor 3 (IRF3) signaling pathway [11, 12]. The intracellular RNA sensors RIG-I/MDA5 also serve as IFN inducers acting via the mitochondrial antiviral signaling protein (MAVS)-IRF3 signaling pathway, thus protecting host cells against the spread of viral invasion [2, 3].

We previously found that the expression of TLR3 and IFN-β mRNAs is significantly increased in both the portal areas and parenchyma of livers diseased with PBC [17]. There was a positive correlation between TLR3 and IFN-β mRNA levels in both areas, indicating that TLR3-type 1 IFN signaling pathway is activated in PBC; the TLR3-expressing and/or IFN-β-producing cells, however, remain unknown [17]. This prompted us to investigate TLR3 expression and IFN-β production in human intrahepatic biliary epithelial cells (HIBECs).

In this study, we used specific monoclonal antibodies against TLRs [4] to determine that intrahepatic bile ducts, but not hepatocytes, in diseased livers strongly express TLR3. TLR3 protein is found in HIBECs at low levels on the cell surface and high levels in endosomes. Our results, however, indicate that the primary signaling pathway for IFN-β induction activated by dsRNA functions via RIG-I/MDA5 in the cytoplasm but not via TLR3 expressed on the cell surface or in endosomes. This is contrary to results obtained for other types of epithelial cells, such as bronchial epithelial cells and endometrial cells, in which surface TLR3 recognizes viral dsRNA to signal the presence of infection via the TLR3-IRF3-type I interferon signaling pathway [9, 11, 12, 15]. Here we discuss dsRNA-sensing system functioning in HIBECs and the role of high expression levels of TLR3 in diseased livers.

Materials and methods

Liver biopsy specimen and immunohistochemical evaluation

Liver needle biopsy specimens, which were derived from seven primary biliary cirrhosis (PBC)-affected, five autoimmune hepatitis (AIH)-affected, and five chronic hepatitis C (CHC)-affected livers, were frozen in OCT compound (Sakura Finetechnical Co, Tokyo, Japan) immediately after the procedure and were stored at −80°C until use. Mouse monoclonal antibodies to human TLR1 (clone TLR1.136, IgG1, *k*), TLR2 (clone TLR2.45, IgG1, *k*), TLR3 (clone TLR3.7, IgG1, *k*), TLR4 (clone TLR4, IgG2a, *k*), and TLR6 (clone TLR6.127, IgG1, *k*) were generated in our laboratory [4]. Among these monoclonal antibodies, the specificity of anti-TLR3 (TLR3.7) was intensively studied. Anti-TLR3 monoclonal antibody specifically binds to the extracellular part of native TLR3 but not to denatured form of TLR3 or other TLRs, including TLR2 and TLR4. Furthermore, TLR3.7 inhibits dsRNA-induced IFN-β production by inhibiting the interassociation between dsRNA and TLR3 [4, 5]. Mouse monoclonal antibodies specific for cytokeratin (CK) 7 and CK 19 were purchased from DAKO (DAKO Japan, Kyoto, Japan). Frozen sections, 4 mm in thickness, were stained with anti-TLR and anti-CK7 or -CK19 antibodies as described elsewhere [17]. Briefly, frozen sections were first fixed in 50 and 100% acetone for 30 s and 3 min, respectively, followed by treatment with Peroxidase Blocking agent (DAKO) for 10 min. Sections were then incubated with anti-TLR monoclonal antibodies (anti-TLR1, 2, 3, 4, and 6) for 60 min at room temperature. A standard 2-step method with ENVISION+ (DAKO) was used to visualize bound antibody using 3,3′-diaminobenzidine as a chromogen