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Mechanism of up-regulation of human Toll-like receptor 3 secondary to infection of measles virus-attenuated strains

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Abstract

PolyI:C, a synthetic double-stranded (ds)RNA, and viruses act on cells to induce IFN-β which is a key molecule for anti-viral response. Although dsRNA is a virus-specific signature and a ligand for human Toll-like receptor 3 (TLR3), largely uncharacterized multiple pathways associate virus-mediated IFN-β induction. Here, we demonstrated that laboratory-adapted but not wild-type strains of measles virus (MV) up-regulated TLR3 expression both in dendritic cells and epithelial cell line A549. The kinetics experiments with the laboratory MV strain revealed that TLR3 was induced late compared to IFN-β and required new protein synthesis. Furthermore, neutralizing antibodies against IFN-β or IFNAR (Interferon-α/β receptor) suppressed MV-induced TLR3 induction, indicating that type I IFN, IFN-α/β, is critical for MV-mediated TLR3 induction. Yet, a recently identified virus-inducible IFN, the IFN-λ, did not contribute to TLR3 expression. A virus-responsive element that up-regulates TLR3 was identified in the TLR3-promoter region by reporter gene experiments. The ISRE, a recently reported site for IFN-β induction, but not STAT binding site, located around −30 bp of TLR3 promoter responded to MV to induce TLR3 expression. This further indicates the importance of type I IFN for TLR3 up-regulation in the case of viral infection. In HeLa and MRC5 cells, augmented production of IFN-β was observed in response to dsRNA when TLR3 had been induced beforehand. Thus, the MV-induced expression of TLR3 may reflect amplified IFN production that plays a part in host defense to viral infection.

Keywords: Toll-like receptor 3; Measles virus; IFN-α/β; ISRE; Transcriptional regulation

Measles is a highly contagious disease responsible for many infant deaths particularly in the developing countries. Although host immune response against measles virus (MV) is usually vigorous, profound immune-suppression is transiently induced by MV. This causes secondary and opportunistic infections [1]. Several complications are also associated with measles [1]. MV infection is mild or nonsymptomatic in vaccinated individuals but immune-suppression occurs even by vaccine strains of MV [2]. The mechanism of the MV-mediated modulation of host immunity has not yet been elucidated.

Entry receptors for MV have been identified as CD46 [3,4] and CDw150/SLAM [5]. CDw150/SLAM is a primary receptor for MV both in wild-type and laboratory-adapted or vaccine strains, while CD46 is a receptor for most of the laboratory strains and some of the wild strains [6]. CDw150/SLAM is a co-stimulator for lymphocyte activation [7]. Both professional APCs (i.e., matured DCs) and B cells are CDw150/SLAM-positive and activation signal is transduced by homophilic adhesion [7]. CD46 is ubiquitously expressed as a receptor for complement C3b [8]. By stimulation with anti-CD46 antibody, C3b or MV, macrophages were found to produce IL-12 and NO [9]. Further, some T cells exhibit

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a regulatory phenotype [10], suggesting the presence of a unique CD46-mediated signaling pathway for T cell differentiation. The relationship between the MV receptors and measles immune modulation still remains largely undefined.

Type I interferon (IFN-α/β) induction is a primary defense system for virus infection [11,12]. Although virus-infected cells are shown to induce IFN-α/β through activation of NF-κB, ATF-2/c-Jun, and IRF-3 in vivo, there have been reported multiple pathways that sustain IFN-α/β production in conjunction with viral infection [13]. Nucleocapsid of MV may activate virus-associated kinase (VAK) [14]. dsRNA is a representative signature pattern specific to viruses and activates cytoplasmic kinases such as PKR that possess RNA-binding domains [15]. Recent reports suggested that Toll-like receptor (TLR) signaling also involves activation of the IFN-β promoter: membrane-expressed TLR3 recognizes dsRNA [16,17] and induces phosphorylation of IRF-3, leading to IFN-β induction via a TLR adapter TICAM-1 or TRIF [18,19]. The adapter TICAM-1 bridges TLR3 and an unidentified molecule that activates IRF-3. Once the primary response allows production of even minute IFN-β, the amplification pathway starts as the secondary response, subsequently provoking IFN-inducible genes [20]. The secondary pathway involves STAT1, STAT2, and IRF-9. IRF-7 is in turn activated to amplify IFN signaling in conjunction with IRF-3 [20,21]. It remains unknown in this positive loop of IFN amplification in cells how virus-derived dsRNA interacts with TLR3.

Using MV strains, human dendritic cells, and epithelial/fibroblast cell lines, here we demonstrated a mechanism whereby TLR3 is up-regulated by infection with laboratory-adapted and vaccine strains of MV. Enhanced production of IFN-β in the TLR3-up-regulated cells revealed that TLR3 is involved in a positive feedback regulation of IFN-α/β signaling pathway.

Materials and methods

Cell culture and reagents. The A549 human lung epithelial cell line and the normal human lung fibroblast MRC5 were obtained from Riken Cell Bank (Tsukuba, Japan). The African green monkey kidney cell line Vero cells was purchased from ATCC. A549, MRC5, and Vero were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS; JRH biosciences, KS) and antibiotics. HeLa was provided by Dr. Fujita (Tokyo Met. Inst.) [19] and kept in MEM containing 5% heat-inactivated FCS and antibiotics. Cells were maintained in 5% CO_2 incubator at 37 °C. Antibodies against IFN-β and IFNAR were purchased from PBL Biomedical Laboratories (NJ). Recombinant IFN-α (OBMPHP107Z) was obtained from Cosmobio (Tokyo, Japan), α-IL10Rβ (MAB874) was from R&D systems (MN). Expression vectors for measles virus proteins, V; pKS-V, and C; pKS-C, and polyclonal antibodies against V or C protein were provided by Dr. Takeuchi in Tsukuba Univ., Tsukuba, Japan [23]. Polyclonal antibodies against CD46 and CDw150/SLAM were produced in our laboratory [6,9]. TLR3.7, a specific monoclonal antibody raised against human TLR3, was previously reported [17].

Immature dendritic cell preparation. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat of normal healthy donors by methylcellulose sedimentation followed by standard density gradient centrifugation with Ficoll–Hypaque (Amersham Bioscience, NJ). For immature dendritic cell (iDC) preparation, CD14+ monocytes were obtained from PBMC by using MACS system (Miltenyi Biotec, Gladbach, Germany) with anti-CD14 mAb-conjugated microbeads and kept in RPMI-1640 (Invitrogen, CA) containing 10% FCS, 500 IU/ml GM-CSF, 100 IU/ml IL-4 (Prepro Tech, London, UK), and antibiotics for 6 days. Morphological changes were examined by phase contrast microscopy (Olympus IX-70, Tokyo, Japan).

Measles virus infection. Measles virus (MV) strains, Nagahata (NV), Masusako (MS), and Edmonston (ED) were obtained from the Institute for Microbial Disease, Osaka University (Osaka, Japan) and the University of Washington (Seattle, USA). The method for preparation of viruses and the properties of MV strains were reported previously [9]. All strains used in this investigation were passaged in Vero cells. Virus containing culture supernatants were tested to confirm that they were free of LPS contamination (<0.2 pg/ml) or mycoplasm lipoprotein (<10 pg/ml). Sufficient amount of medium that covers the monolayers to avoid damage to cells was carefully monitored during viral infection. Infection with virus was conducted for a period of 1.5 h.

Reporter construction. The BAC clone, RP11-279K24, bearing TLR3 genomic sequence was purchased from BACPAC resources (CA). DNA was prepared by alkaline-SDS method and used as template for PCR amplification of various sizes of upstream region of human TLR3 gene. DNA fragments containing upstream sequence and promoter region of human TLR3 were obtained by 15 cycles of PCR with LA-*Taq* DNA polymerase (TaKaRa, Kyoto, Japan). Oligonucleotides were synthesized at Hokkaido system science (Hokkaido, Japan). Forward primers used were 5′-GGGGTACCATG ACTCAATGCAAACTTG AATTTGCC-3′ (pu2.0), 5′-GGGGTACC AGAGCCATTCATTAGTCAACCAAAG-3′ (pu1.5), 5′-GGGGTA CCTATAAAGCGGTCTA GCTGAAGCTGGAG-3′ (pu1.0), and 5′-GGGGTACCTCTCAGCTTTGCCATGTTTGGC-3′ (pu0.5 termed as kp500F), respectively, and the reverse primer used was 5′-GAA GATCTCATTTCAGGGAAGTGTGTGGC-3′. PCR products were digested with *Kpn*I and *Bgl*II and subcloned into upsteam of Luciferase gene in pGV-E2 plasmid (TOYO B-Net, Tokyo, Japan). Each construct was made from two independent PCR fragments to confirm that the results were not due to errors caused during PCRs.

To identify the virus-responsive enhancer element in 0.5 kb upstream from TLR3 promoter, pu0.5 was subcloned into pGL3-promoter plasmid (Promega, WI) and termed as pGL0.5. Inserts of 3′ deletion version of pGL0.5, the pGL0.5d30, and the pGL0.5d80 were amplified by PCR as described above using kp500F as forward primer and 5′-GAAGATCTGCACTCTCGAAA GTGAAAGTAAAGCTT-3′ and 5′-GAAGATCTGTGCATTCCAAGAAGGGCAAAG-3′ as reverse primers, respectively. Mutations in STAT binding site and ISRE were made by PCR with high fidelity *Taq* polymerase, Pyrobest (TaKaRa, Kyoto, Japan). Primers for mutation of STAT binding site were 5′-CTCCCTTTGCCCCCCTTGGAATGCAC-3′ and 5′-GTGC ATTCCAAGGGGGGCAAAGGGAG-3′, for ISRE mutation were 5′-GCTTTACTTTCACGATCGAGAGTGCCG-3′ and 5′-CGGCA CTCTCGATCGTGAAAGTAAAGC-3′ using pGL0.5 as template. PCR products were digested with *Dpn*I (New England Biolabs, MA) at 37 °C for 1 h and 1 μl of digested product was directly transformed to DH5α. Mutations were confirmed by sequencing and mutated 0.5 kb inserts were subcloned into *Bgl*II/*Kpn*I site of pGL3 promoter.

Transfection and Luciferase assay. The day before transfection, A549 cells were plated at the density of 4×10^5/well onto a 6-well culture plate (#353046, Becton–Dickinson, NJ). Plasmid DNA was incorporated into cells with Lipofectamine PLUS (Invitrogen, CA) according to manufacturer's instructions. Sixteen to twenty-four hours after transfection, cells were treated with trypsin and re-plated onto a

24-well culture plate. Viral stimulation was mainly done with MV at m.o.i. = 0.1 for 24 h. After stimulation, cells were harvested and lysed with Promega reporter lysis buffer (Promega, WI). Luciferase and β-galactosidase activities were measured by PicaGene luciferase assay system (TOYO B-Net, Tokyo, Japan) and β-Galactosidase enzyme assay system (Promega, WI), respectively. Promoter activity was calculated by normalizing the luciferase activity with β-galactosidase activity.

Cell lysate preparation, SDS–PAGE, and Western blotting. Cells were harvested by scraping and washed twice with PBS. Lysis buffer containing 0.1% NP40 and protein inhibitors in PBS was added to the cell pellet, suspended well, kept on ice for 15 min, and centrifuged at 15,000 rpm for 10 min at 4 °C. Protein concentration of the supernatant was measured by Bio-Rad protein assay kit (Bio-Rad Laboratories, CA). Lysate containing 30 μg protein was separated on denaturing SDS–PAGE and transferred to PVDF membrane (Immobilon-P; Millipore, MA). Membranes were blocked in 5% skim milk in PBS for 30 min at room temperature and then blotted with specific polyclonal antibodies diluted in 2% skim milk for 1 h. After washing with PBS, HRP-conjugated secondary antibody (Biosource, CA) diluted 1:10,000 was applied on membranes, incubated for 30 min, and washed with 0.1% Tween 20 in PBS. Detections were performed with ECL detection kit (Amersham Bioscience, NJ).

Determination of IFN-β level. Culture media were centrifuged to remove cell debris and supernatants were stored at −80 °C until the assay. The level of secreted IFN-β in the culture medium was determined with ELISA kit (FUJIREBIO, Tokyo, Japan) for human IFN-β according to the manufacturer's protocol.

Flow cytometry. Cells were harvested by treating with 10 mM EDTA in PBS at 4 °C for 15 min. For intracellular staining, cells were suspended in 1× FACS permeabilizing solution (Becton–Dickinson, NJ) and kept in dark for 10 min at room temperature. TLR3.7 monoclonal antibody was used for human TLR3 staining at the concentration of 1 μg/ml and mouse IgG1 (MOPC-21, Sigma, MO) was used for subclass control at the same concentration. After incubation on ice for 30 min, cells were washed with PBS containing 0.2% BSA, and 0.1% sodium azide and re-incubated with FITC-conjugated goat anti-mouse IgG (American Qualex, CA) for 30 min on ice. Cells were washed as above and fluorescence intensity was measured by flow cytometric analysis (FACSCaliber, Becton–Dickinson, NJ).

RT-PCR and quantitative PCR. A549 cells were harvested by trypsin treatment. DCs were collected by centrifugation. Total RNA was extracted with RNeasy mini kit (Qiagen, GmbH, Germany). Two micrograms of total RNA was incubated at 70 °C for 5 min and kept on ice for 2 min, and reverse transcription was performed with MMLV-reverse transcriptase (Promega, WI) at 37 °C for 90 min followed by PCR amplification. PCR primer sequences are shown in Table 1. PCR for quantitative PCR was done with iQ SYBER Green Supermix and amplified PCR products were measured by iCycler iQ real-time PCR analyzing system (Bio-Rad laboratories, CA).

Table 1
Sequence of primers used for RT-PCR

gene	primer sequence
TLR3	5′-CTCAGAAGATTACCAGCCGCC-3′ 5′-CCATTATGAGACAGATCTAATG-3′
IFN-β	5′-ATTGCCTCAAGGACAGGATG-3′ 5′-GGCCTTCAGGTAATGCAGAA-3′
MxA	5′-CCACTGGACTGACGACTTGA-3′ 5′-GAGGGCTGAAAATCCCTTTC-3′
IP10	5′-CCACGTGTTGAGATCATTGC-3′ 5′-GCAGGGTCAGAACATCCACT-3′
H protein	5′-TCAGTAATGATCTCAGCAACTG-3′ 5′-TTCAATGGTGCCCCACTCGGGA-3′
GAPDH	5′-CACAGTCCATGCCATCACTG-3′ 5′-TACTCCTTGGAGGCCATGTG-3′
IFN-λ1	5′-AAGCCCACCACAACTGGGAA-3′ 5′-AAGGTGACAGATGCCTCCAG-3′
IFN-λ2,3	5′-TAGCCCAGTTCAAGTCCCTG-3′ 5′-AGAGGATATGGTGCAGGGTG-3′
ISG15	5′-TGTCGGTGTCAGAGCTGAAG-3′ 5′-GCCCTTGTTATTCCTCACCA-3′
OASL	5′-CTTCGGGAATGGCACGGTTC-3′ 5′-CAGCAGGTCCTGGCTTTGCC-3′

Results

TLR3 mRNA expression was induced upon MV infection

Human immature DCs (iDCs) were infected with wild-type (MS) and attenuated strains of MV (NV, ED). Twenty-four hours post-infection (h p.i.), the levels of TLR3 mRNA were assessed by RT-PCR. NV and ED efficiently induced TLR3 signal whereas MS barely induced TLR3 mRNA (Fig. 1A). This, together with accumulating results on IFN-inducing ability of MV strains [24], suggested that attenuated laboratory strains induce TLR3 expression while wild-type strains do not. These MV strains are known to have different receptor preferences to enter host cells as shown in Table 2 [6]. iDCs express both CD46 and CDw150/SLAM as entry receptors on the cell surface (Fig. 1C) and indeed the H protein, which is encoded in the virus genome, was generated in iDCs infected with each of these three strains (Fig. 1A). Thus, lesser TLR3 induction by MS infection does not depend on the receptor-defined tropism in host cells.

Similar induction of TLR3 mRNA after MV infection was also observed in human lung epithelial cell line A549. At 24 h p.i., NV and ED markedly induced TLR3 mRNA (Fig. 1B, lanes 3 and 4) while MS failed to activate TLR3 transcription (Fig. 1B, lane 5). Unlike in DCs, CDw150/SLAM was not detectable in A549 cells by Western blotting (Fig. 1C). Therefore, a weak expression of H protein in A549 cells suggests that MS barely, if at all, infected these cells.

Thus, the TLR3 gene is expressed upon MV infection in a strain-specific manner. Of the strains tested, NV induced a robust signal of TLR3, which was detected even 10 h p.i. in A549 cells (Fig. 1B, lane 2). We used the NV strain for further study on TLR3 up-regulation.

Two phase induction of IFN-β in DC by NV infection

Virus infection rapidly induces type I IFNs, IFN-α/β, and this primary response does not require new protein synthesis. Primarily induced type I IFNs are then

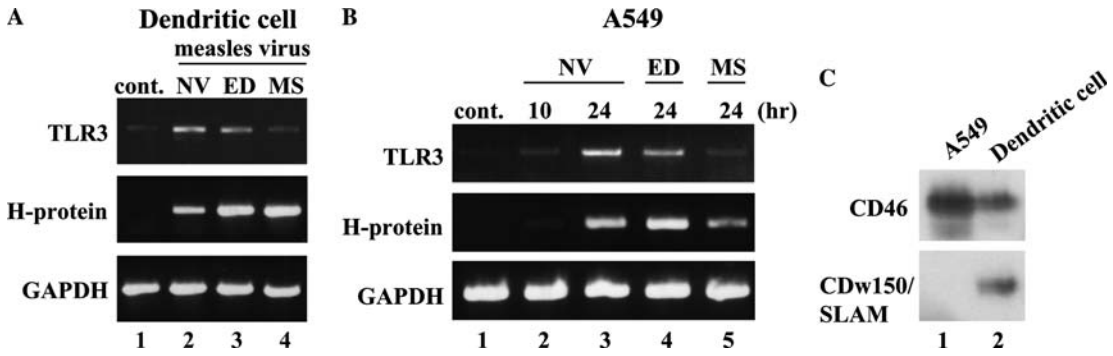

Fig. 1. Induction of TLR3 by measles virus infection. (A) Immature dendritic cells (iDCs) were infected with three different strains of measles viruses (Nagahata (NV); lane 2, Edmonston (ED); lane 3, and Masusako (MS); lane 4) at multiplicity of infection (m.o.i.) of 0.5 for 24 h. iDCs left uninfected were controls (lane 1). Cells were harvested by brief centrifugation and washed twice with PBS. Total RNA was prepared and the expression level of TLR3 was determined by RT-PCR. Gene-specific PCR primer sets for GAPDH and H protein were used as internal control and monitoring virus replication, respectively. One of the three similar experiments is shown. (B) Human lung epithelial cell line A549 cells were infected with MV strains used in panel A at m.o.i. = 0.5 for indicated hours. Cells were harvested by treating with trypsin and total RNA was extracted. RT-PCR was performed as in panel A. One of the three similar experiments is shown. (C) Whole cell extracts were prepared from A549 (lane 1) and iDC (lane 2) growing under normal condition. Cell extract containing 30 μg of proteins was subjected to 10% SDS–PAGE and transferred to PVDF membrane. Then membranes were probed with polyclonal antibody (diluted to 1:1000) against CD46 (upper panel) or CDw150/SLAM (lower panel).

Table 2
Receptor usage of measles virus strains on DC and macrophages

	CD46	CDw150/SLAM
NV	+	–
ED	+	+
MS	–	+

DCs and macrophages endogenously express two major measles virus entry receptors, CD46 and CDw150/SLAM. ED strain uses both CD46 and CDw150/SLAM while MS strain uses CDw150/SLAM for its entry. NV enters cells via CD46 and SLAM judging from the results using CHO and HeLa cells expressing SLAM (Taniguchi et al., unpublished data), but in macrophages and DCs, NV of CDw150/SLAM-dependent entry could not be detected [6]. These strain-specific receptor usages were previously determined by competition analysis with specific polyclonal antibodies against CD46 or CDw150/SLAM [6].

secreted and bind their receptor (IFNAR). This leads to amplification of its signal by up-regulating the expression of a set of genes called IFN-stimulated genes (ISGs) to develop anti-viral response [11]. Together with the expression of TLR3, we also examined the levels of IFN-β and MxA expression which are marker genes of primary and secondary response, respectively.

At timed intervals total RNA was extracted from MV-infected iDCs and analyzed by RT-PCR and real-time PCR (Fig. 2). iDCs were prepared from two healthy individuals. In iDC from donor A, two peaks of the IFN-β signal were detected at 3 and ~22 h p.i. of MV. In comparison with IFN-β, MxA was induced later than 3 h p.i. nearly paralleling the late induction. The kinetics of TLR3 mRNA induction was more retarded than that of MxA, emerging later than 6 h p.i. which was confirmed by quantitative PCR (Fig. 2, donor A). Similar tendencies were observed in iDC from donor B, although the cells were less sensitive to MV than those of donor A. In both iDCs, MV clearly induced TLR3 later than IFN-β.

TLR3 induction is the secondary response of MV infection

We used the A549 cell line to compare the results observed in iDCs. Time course experiments demonstrated that a weak but distinctive induction of IFN-β was detected at 2 h after MV infection (Fig. 3A). A slight decrease of IFN-β mRNA at 1 h was observed reproducibly in independent experiments (data not shown). In contrast to IFN-β, induction of MxA and TLR3 occurred at later time points, requiring 6 h p.i. for detection. Thus, the kinetics of induction of IFN-β/TLR3 in MV-infected A549 cells resembled that of iDCs.

In order to test the requirement of new protein synthesis on this MV-induced TLR3 expression we used protein synthesis inhibitor cychlohexamide (CHX). IFN-β mRNA was detected at 8 h p.i. of MV while that of MxA was strongly suppressed in the presence of CHX (Fig. 3A, lane 8). This result is consistent with the concept that up-regulation of viral-induced secondary-responsive genes requires the synthesis of IFN-α/β and transcriptional factors [11,13]. MxA is a secondary-responsive gene and so was TLR3. Hence, the induction of TLR3 mRNA by MV is a secondary response of the infection and requires long-term for newly protein synthesis.

Involvement of IFN-α/β in MV-induced TLR3 expression was further confirmed with recombinant IFN-α (rIFN-α) which shares the same receptor as IFN-β. rIFN-α induced TLR3 in A549 cells in a dose and time dependent manner (Fig. 3B). The TLR3 induction by rIFN-α was observed within 2 h after incubation, faster

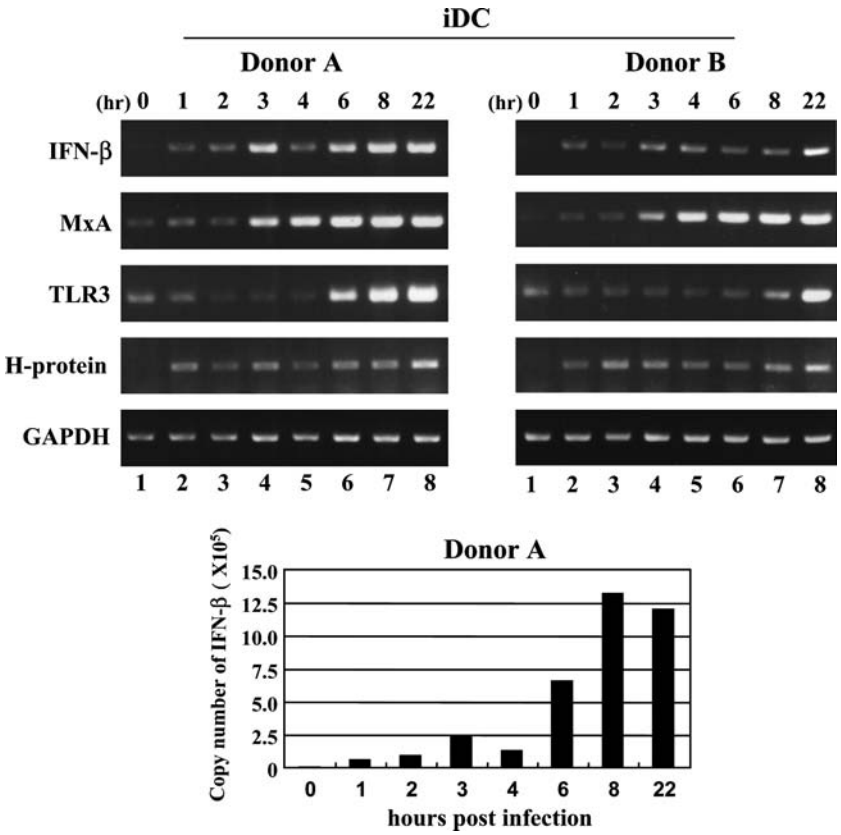

Fig. 2. Kinetics of TLR3 induction following MV infection in DC. iDCs were infected with MV (NV strain) at m.o.i. = 0.5. Cells were harvested at indicated time points and expression levels of the primary- and secondary-responsive genes were analyzed by RT-PCR as in Fig. 1. Experiments were performed with iDCs prepared from two healthy donors. IFN-β mRNA levels in cDNA samples from donorA at each time point were quantified by iCycler iQ real-time PCR system (lower panel).

than that of MV infection. Then, we tested if the blocking of type I IFN signaling pathway affects TLR3 expression in MV-infected cells by using neutralizing antibodies. TLR3 was induced after MV infection in the presence of control IgG (Fig. 3C, lanes 1 and 2) but this was suppressed when IgG was substituted with blocking antibodies against either IFN-β or its receptor, IFNAR (Fig. 3C, lanes 3 and 4). The results were consistent with those obtained by quantitative real-time PCR analysis. Thus, MV infection induces the expression of TLR3, which in part relies on type I IFNs secreted from host cells. TLR3 is one of the ISGs which appears to function to protect host cells from viral invasion.

Recently, a novel family of IFNs, IFN-λ (IFN-λ1/IL29,IFN-λ2/IL28A, and IFN-λ3/IL28B), was found to be up-regulated by viral infection [25,26]. They interact with a heterodimeric class II cytokine receptor that consists of IL-10Rβ and CRF2-12/IL-28R. IFN-λ induces STAT1 activation, suggesting that IFN-α/β and IFN-λ use similar signaling mechanisms to activate gene transcription. In this study, MV infection, but not IFN-α, caused obvious induction of IFN-λ genes in A549 cells (Fig. 3D). Then we tested whether antibody-blocking of IL-10Rβ inhibits the MV-mediated TLR3 expression by RT-PCR along with quantitative real-time PCR (Fig. 3E). Anti-IL-10Rβ barely inhibited MV-mediated TLR3 mRNA induction while anti-IFNAR reproducibly suppressed TLR3 induction (Figs. 3C and E). We further analyzed the effect of anti-IL-10Rβ on the MV-induced expression of other ISGs which showed no change.

MV-responsive element in the TLR3-promoter region

Next we investigated the *cis*-acting element(s) in the TLR3-promoter region that responds to virus infection. For this purpose 2 kb fragment up-stream from 5′ end of the reported TLR3 exon 1 was subcloned into a reporter plasmid (termed as pu2.0), transferred into A549, and then cells were stimulated with MV. The results demonstrated an increased basal expression of reporter luciferase gene. Luciferase activity was up-regulated about 2.5-fold after 24 h p.i. of MV (Fig. 4A). Thus, both promoter activity and virus-responsive element(s) of TLR3 are localized within this 2 kb up-stream (−2.0 kb) region. Serial deletions from 5′ end of this 2 kb fragment demonstrated that −0.5 kb sequence (pu0.5) was sufficient for virus responsibility as well as promoter activity (Fig. 4A).

Search for putative transcription factor binding sites within this −0.5 kb of TLR3 gene through TRANS-

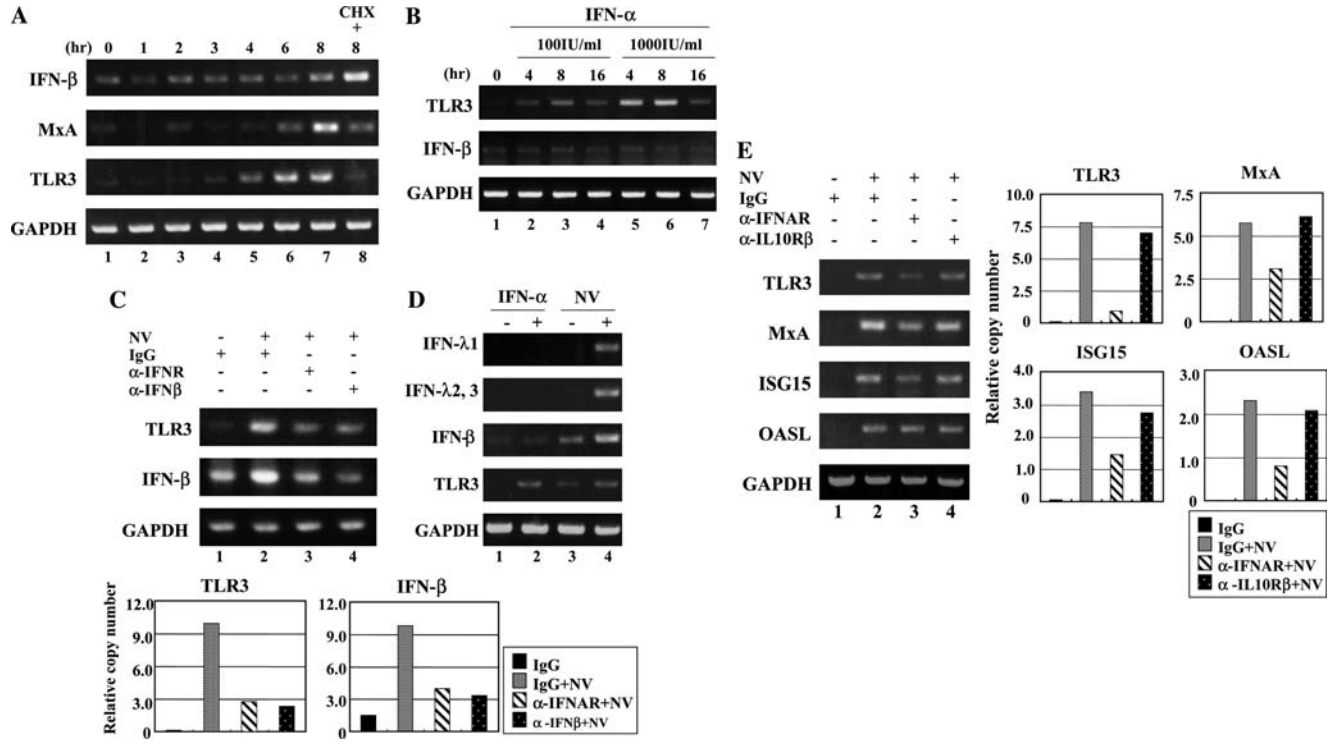

Fig. 3. Effect of MV infection or type I IFNs in TLR3 expression. (A) A549 cells were infected with MV at m.o.i. = 0.1 for indicated time periods (lanes 1–8). Protein synthesis inhibitor cychlohexamide (CHX) (100 μg/ml) was added to the medium 30 min prior to virus infection and kept in the medium during MV infection for 8 h (lane 8). Cells were harvested and expression levels of IFN-β, MxA, and TLR3 were determined by RT-PCR. A representative result out of four trials is shown. (B) Recombinant IFN-α was added at low (100 IU/ml) and high (1000 IU/ml) concentrations to A549 cells and cells were cultured for indicated periods. Total RNA was extracted and RT-PCR was performed as in (A). (C) A549 cells were pretreated with control mouse IgG (50 μg/ml; lanes 1 and 2), α-IFNAR (20 μg/ml; lane 3), or α-IFN-β (2000 NU/ml; lane 4) for 1 h. NV infection was done at m.o.i. = 0.1 for 20 h. Cells were harvested and the expression levels of TLR3 were compared by RT-PCR. mRNA levels were also assayed by quantitative real-time PCR. Experiments were repeated and typical results were presented in relative expression units. (D) Expression levels of IFN-λ 1–3 before (lanes 1 and 3) and after IFN-α treatment (1000 IU/ml for 2 h; lane 2) or NV infection (m.o.i. = 0.1 for 20 h; lane 4.) in A549 cells. RT-PCR analysis was performed with primers for the indicated genes. Notice that IFN-λ2 and IFN-λ3 could not be distinguished since they highly resembled each other. (E) A549 cells were pretreated with control IgG (lanes 1 and 2), α-IFNAR (lane 3), or α-IL10Rβ (lane 4) for 30 min before NV infection. NV infection was done at m.o.i. = 0.1 for 16 h in the presence of antibodies. Total RNA was prepared and expression levels of indicated ISGs were determined by RT-PCR and quantitative real-time PCR.

FAC database revealed that the binding site for NF-κB was found at −0.3 kb of TLR3 gene. However, deletion of this site did adversely affect either both promoter activity or virus response (data not shown). Putative binding sites for STAT family and ISRE (interferon-stimulated response element) were found within −100 bp of TLR3 promoter (Fig. 4B). To analyze the virus responsibility of these sites, 3′-deleted fragments were subcloned into pGL3-promoter plasmid in which expression of luciferase reporter gene is driven by SV40 promoter (Fig. 4C). A deletion construct termed pGL0.5d30 had comparative virus responsibility to the parental construct pGL0.5. Further deletion termed pGL0.5d80 in which the entire STAT binding site was still intact while the ISRE has been deleted did not respond to MV infection. Point mutation within either ISRE or STAT binding site supported this finding. Mutations in ISRE (TTTCAC**TT**T → TTTCAC**GA**T) caused loss of viral induction of luciferase activity while those in STAT binding site (CA**TT**CCAA → CA**GG**CCAA) had no effect on virus responsibility (Fig. 4C). These results suggest that the ISRE at −30 bp confers virus-mediated activation of TLR3 promoter.

V protein of MV inhibits virus-induced TLR3 expression

According to recent reports, V protein of some paramyxoviruses has an inhibitory effect on STAT1 activation [23,27–29]. For example, V protein of mumps virus degrades IFNAR-associated STAT1 and subsequently inactivates the downstream signals from IFNAR [27]. V protein of wild-type MV also interferes with the IFNα/β signaling by inhibiting the phosphorylation of both STAT1 and STAT2 [23].

Our observation thus far suggested that MV-induced TLR3 expression is downstream of IFNAR. rIFN-α indeed activated the pu0.5 TLR3 promoter (data not shown). To confirm this, an expression plasmid for V protein of wild-type MV (MV-V) was transferred

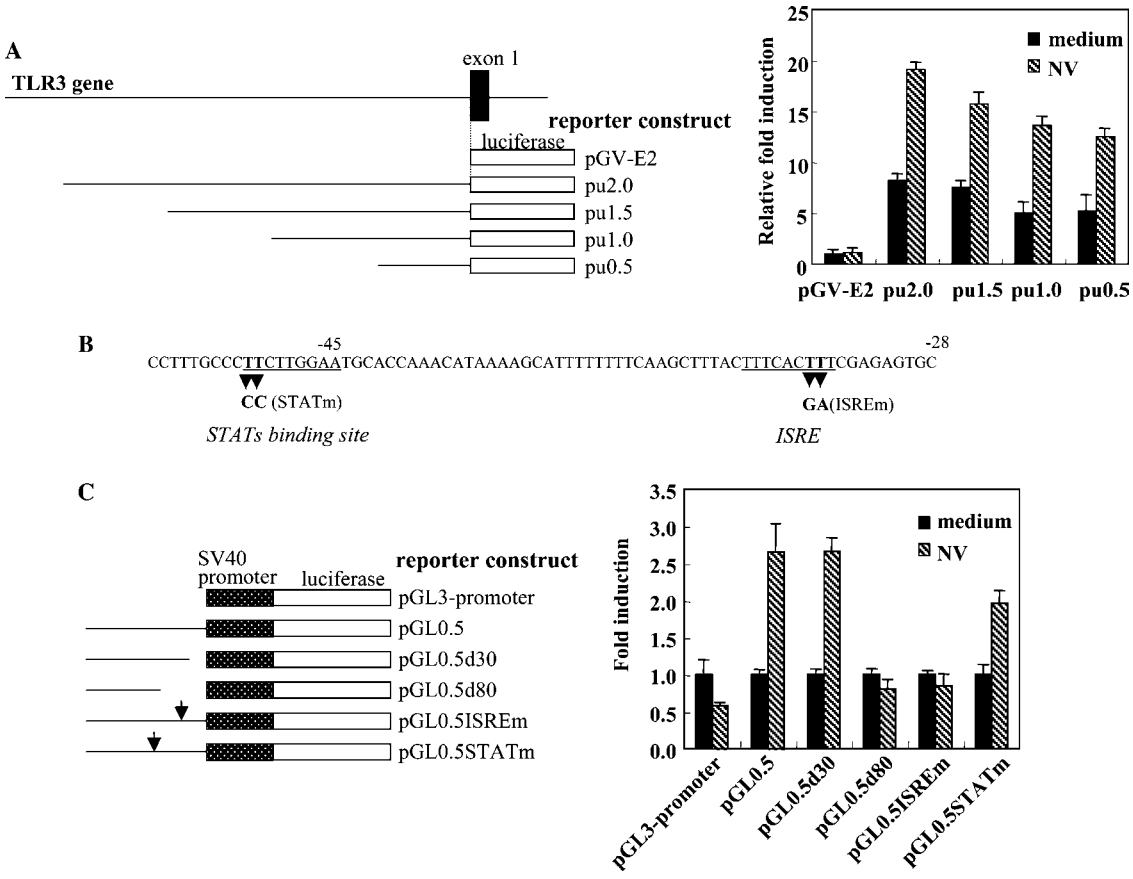

Fig. 4. MV-responsive element in the promoter region of TLR3. (A) Reporter constructs (0.8 μg/well) having different lengths of TLR3-promoter region in upstream of luciferase reporter gene were transferred into A549 cells with internal control plasmid (β-actin–β-Gal., 0.2 μg/well). Sixteen to twenty-four hours post-transfection, cells were treated with trypsin and re-plated for stimulation. NV was infected at m.o.i. = 0.1 for 24 h and cell extracts were prepared. Luciferase activity was measured and normalized to activity of β-galactosidase. Relative fold induction against mock transfectant (pGV-E2) stimulated with medium is indicated. The same assay was performed three times and one of the typical results is presented. (B) DNA sequence around −0.1 kb of human TLR3-promoter region. Putative STAT binding site and ISRE are underlined. Mutated nucleotides are indicated by bold letters. (C) Detailed analysis of −0.1 kb of TLR3-promoter region. Deletion mutants from 3′ and point mutations in ISRE or STAT binding site were made and subcloned into pGL3-promoter vector. Reporter assay was performed as in (A). Virus-mediated reporter induction of each reporter construct was determined as fold activation of normalized luciferase activity of MV infected (solid bar) vs. medium treated (hatched bar). One of the three experiments is shown.

into the cells with the pu0.5 reporter plasmid (Fig. 5A). The pu0.5 construct failed to induce MV-dependent luciferase if MV-V was concomitantly expressed. On the other hand, neither C (MV-C) nor control plasmid showed significant effect on MV-mediated luciferase induction (Fig. 5A). Western blotting analysis revealed that significant expression of V and C proteins in the reporter gene assay (Fig. 5B). Hence, MV infection induces type I IFN followed by TLR3 expression: this process requires STAT1 and/or STAT2 activation by IFNAR.

Increased expression of TLR3 enhanced polyI:C response

To further illustrate the biological significance of this TLR3 induction, we tested whether TLR3 synthesized in response to viral infection modulates the response against dsRNA such as polyI:C (Fig. 6). Once polyI:C binds the extracellular domain of TLR3, it strongly induces the production of IFN-β through TICAM-1 (or TRIF) [18,19,30]. A549 cells, however, hardly responded to polyI:C when this TLR3 ligand was added to culture medium and this may correlate with restricted intracellular staining of TLR3 protein in this cell line (data not shown). Thus, HeLa and MRC5 cells were used for this study to resolve this issue.

The HeLa cell subline we used responded to polyI:C and produced IFN-β [17]. This cell line, however, was resistant to MV by yet unknown reasons (data not shown). Up-regulation of TLR3 in response to IFN-α was examined using this HeLa subline and expected results were obtained in terms of TLR3 mRNA level (Fig. 6A, lanes 1 and 3) and protein level (Fig. 6B, left lane). Similar results were obtained with IFN-α-stimulated MRC5 cells (data not shown). In MRC5 cells, MV infection caused up-regulation of TLR3 message

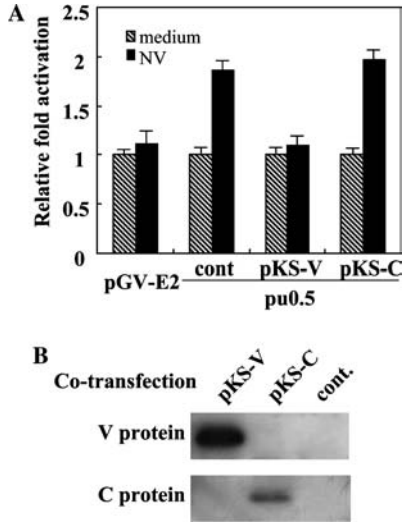

Fig. 5. Effect of MV-V and MV-C on TLR3 up-regulation. (A) A549 cells were transfected with expression vector (0.9 μg/well) for measles virus V protein (pKS-V), C protein (pKS-C) or control plasmid (cont) together with pu0.5 (0.8 μg/well) and internal control plasmid (β-actin–β-Gal; 0.2 μg/well). Sixteen hours after transfection, cells were re-plated and MV infection was done at m.o.i. = 0.1 for 24 h. Cell lysates were then collected and luciferase and β-galactosidase activities that were measured as described in the text. Relative fold activation was determined as in Fig. 4C. One of the three experiments is shown. (B) Western blotting analysis of protein expression of co-transfected V or C proteins. Thirty micrograms of whole cell lysate protein was subjected to 12.5% SDS–PAGE, transferred to PVDF membrane, and probed with polyclonal antibodies against to V or C protein.

(Fig. 6A, lanes 5 and 7), which was accompanied by increased surface protein level (Fig. 6B).

IFN-β levels in culture medium of the relevant cells were measured by ELISA (Fig. 6C). In both cell lines, IFN-β in the medium was increased even under normal conditions in response to polyI:C whereas TLR3 was not induced (Fig. 6B, "cont."). We previously demonstrated that induction of IFN-β secretion by polyI:C was partially inhibited with monoclonal-antibody specific for TLR3, TLR3.7 [17]. Thus, this response is, at least partially, dependent on surface expression of TLR3. In both HeLa and MRC5 cells, increased TLR3 expression resulted in enhanced production of IFN-β in response to polyI:C. In MRC5 cells, MV infection per se resulted in an increase of IFN-β level (Fig. 6C, solid bar on "NV" in MRC5), MV-infected MRC5 cells further responded to polyI:C (from 15.6 to 23.5 IU/ml of IFN-β).

These results demonstrated that increased TLR3 expression results in enhanced production of IFN-β in response to polyI:C stimuli. This may reflect the requirement of TLR3 and its function in virus infection.

Discussion

In the present study, we have demonstrated that certain MV strains induce TLR3 expression in human DCs and other cell lines. The results also show direct involvement of type I IFN in this induction and, indeed, the ISRE at −30 bp of TLR3 promoter participates in virus-mediated TLR3 expression. In HeLa and MRC5 cells levels of TLR3 expression were elevated by exogenously added IFN-α which caused higher IFN-β production in response to dsRNA stimulation. Thus, we conclude that TLR3 is one of the ISGs which is up-regulated in a positive feedback fashion in virus-infected cells. It has been speculated that TLR3 is implicated in host defense against virus infection by its dsRNA response [31–33]. Our finding on the viral induction of TLR3 expression may be associated with protective immunity against RNA viruses.

It is well known that MV induces profound host immune-suppression. Naniche et al. [24] first reported the finding that wild-type MV strains, but not vaccine and laboratory-adapted strains, interfere with the induction of type I IFN, thereby circumventing host anti-viral immunity. One possibility is that MV-mediated immune-suppression occurs in conjunction with defective IFN induction by wild-type MV strains. However, immune-suppression is more or less induced by wild-type as well as vaccine strains of MV, which declines the idea that

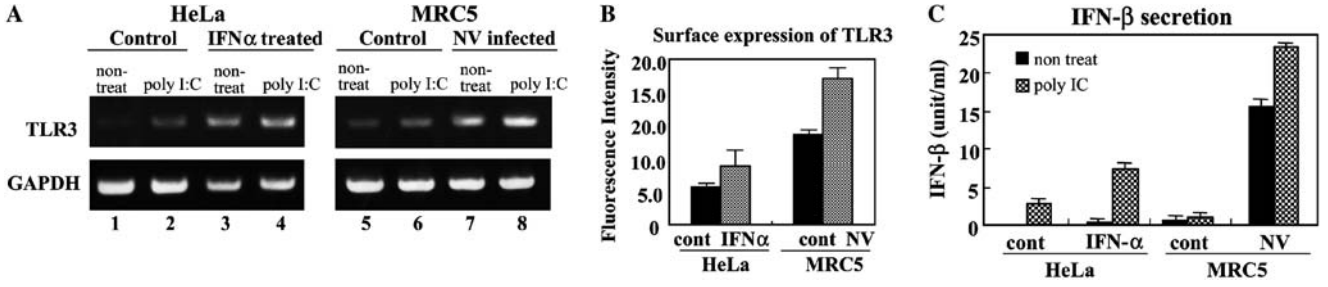

Fig. 6. Expression level of TLR3 in response to polyI:C. (A) Induction of TLR3 in HeLa and MRC5 cells by IFN-α (500 IU/ml for 2 h) or NV infection (m.o.i. = 2.5 for 24 h), respectively. TLR3 mRNA levels before and after induction were assessed by RT-PCR. GAPDH was used as a control. (B) Flow cytometric analysis of cell surface expression levels of TLR3. Geographic mean value of fluorescence reflecting the TLR3 protein expression level is indicated. One of the three experiments is shown. (C) The level of IFN-β secreted into culture medium. HeLa cells expressing TLR3 in response to IFN-α or MRC5 cells expressing TLR3 via NV infection were stimulated with 10 μg/ml polyI:C for 6 h. Then, the culture medium was collected, centrifuged to remove cell debris, and kept at −80 °C until the time for assay. IFN-β levels were measured by ELISA.

immune-suppression reflects low viral potency of IFN induction in host cells. Indeed, all MV vaccine strains tested to date efficiently induce IFN-β as well as mild immune-suppression [24]. Some MV vaccine strains also exhibit poor replication properties in human DCs [6]. It is likely that the IFN-inducing properties of MV are related to virus attenuation in host cells. Nonetheless, we could not detect any contribution of IFN-λ to virus-activated expression of ISGs including TLR3 in A549 cells.

The 0.3 kb TLR3-promoter region was responsible for virus-enhanced promoter activity. Besides the ISRE at −0.1 kb, we observed that the Sp1 binding site at −0.2 kb is also responsible for both virus-mediated up-regulation and basal expression of TLR3. Sp1 consists of GC-rich sequences which direct transcription of TATA-less promoter and are also known to make up for the absence of TATA-box for transcription initiation [34,35]. A regulatory profile similar to TLR3 was reported in ISG20 gene expression [36]. There are several transcriptional starting sites for human TLR3 [37], a characteristic feature of GC-rich sequence-driven gene transcription [38]. In human TLR3 promoter, we identified an ISRE virus-responsive element and mutation of the ISRE resulted in dramatic decrease of promoter activity. The ISRE of human TLR3 appears to be involved in both maintenance of promoter integrality and promoter-specific virus-responsive element.

TLR3 has been thought to participate in provoking anti-viral immunity as it recognizes polyI:C that induce IFN-β expression [20]. In the present study, we demonstrated that MV strains with IFN-β inducible properties up-regulate TLR3 expression following their infection. Increased TLR3 expression level resulted in enhanced IFN-β secretion in response to polyI:C. This is consistent with the implicated function of TLR3 and also suggests the existence of positive feedback regulation of IFN-α/β signaling in response to virus infection [22]. It is possible to surmise that host cells have developed such a positively activated pathway to establish an effective anti-viral environment. IFN-α/β has been reported to confer unique maturation properties on DC [39,40], and in viral infection, IFN-induced TLR3 may play a major part in this specific DC maturation. In this scenario, however, unsettled points are where virus-derived dsRNA encounters TLR3 and how TLR3-TICAM-1 signaling induces DC maturation.

We finally assigned the relevant ISRE in the TLR3 promoter as a virus-responsive element as well as the site for IFN-β response. These results reinforce the importance of type I IFN secreted by virus infection for TLR3 induction. IRF-1 was demonstrated to be a transcriptional activator which binds to the ISRE of human TLR3 following type I IFN stimulation [40,41]. Activation of IRF-1 is regulated mainly at transcriptional level and homodimerized STAT1 binding to the responsive element called GAS in IRF-1 promoter is important for the induction [41,42]. For dimer formation, phosphorylation of STAT1 is required. V protein of wild-type MV suppressed the phosphorylation of STAT1 and STAT2 [23]. Thus, it is likely that the effect of MV-V protein on TLR3 induction in the reporter assay is due to the inability of homodimerization of activated STAT1 with consequent loss of IRF-1 transcription.

We detected TLR3 protein only inside the DC and A549 cells. Such intracellular localization of TLR3 was also observed in other cell types [39]. The route by which viral dsRNA replicating in cytoplasm encounters intracellular TLR3 still remains unknown. In light of our study it would be interesting to clarify the virtual function of intracellular TLR3 and the molecular mechanism whereby anti-viral immunity occurs through the TLR3 pathway inside the cells. Studying this issue in association with activation of the reported anti-viral agents including PKR is in progress in our laboratory.

Acknowledgments

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